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Inhibitory effect of *Zingiber cassumunar* extracts on lipopolysaccharide-induced cyclooxygenase-2 and matrix metalloproteinase expression in human gingival fibroblasts

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Background and Objective: Lipopolysaccharides (LPS) induce the production of proinflammatory mediators such as prostaglandins and matrix metalloproteinases (MMPs) in human gingival fibroblasts (HGFs). *Zingiber cassumunar* is a medicinal plant that possesses anti-inflammatory properties. The aim of this study was to determine the effects of the *Z. cassumunar* extract on the expression of cyclooxygenase (COX)-1, COX-2 and MMP-2 in HGFs challenged with LPS.

Material and Methods: HGFs were treated with LPS in the presence or absence of *Z. cassumunar* extracts. The levels of expression of *COX-1*, *COX-2* and *MMP-2* mRNAs and of COX-1, COX-2 and MMP-2 proteins were detected by reverse transcription–polymerase chain reaction and western blotting, respectively. MMP-2 activities in cell-culture supernatants were determined using gelatin zymography. MAPK activation was evaluated by western blotting.

Results: LPS treatment of HGFs resulted in the activation of ERK1/2, p38 and JNK. *Z. cassumunar* extracts significantly inhibited the phosphorylation of ERK1/2 and JNK in HGFs stimulated with LPS. A lesser inhibitory effect was observed for the phosphorylation of p38. RT-PCR and western blot analyses showed that *Z. cassumunar* extracts inhibited the LPS-induced expression of *COX-2* mRNA and COX-2 protein, respectively, but not of *COX-1* mRNA or COX-1 protein. Pretreatment of HGFs with *Z. cassumunar* also attenuated the induction of MMP-2 with LPS.

Conclusion: Our results indicate that *Z. cassumunar* extracts inhibit COX-2 and MMP-2 production by LPS-activated human gingival fibroblasts through blocking the proinflammatory signaling pathway involving ERK1/2, JNK and p38.

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Periodontal disease is one of the most prevalent oral diseases. The disease is caused by ecological changes of bacteria in dental biofilm, which lead to inflammation of the gingiva and destruction of periodontal tissues (1). The tissue destruction is the result of activation of the host immune –inflammatory responses to virulence factors (2). One of the main virulence factors is the lipopolysaccharide (LPS) component of the cell wall of gram-negative bacteria. LPS obtained from periodontal pathogens has been proposed to contribute to alveolar bone loss and connective tissue degradation in periodontal disease (3).

Matrix metalloproteinases (MMPs) are a family of proteases responsible for degrading components of extracellular matrixes. These enzymes have similar structural and biochemical properties, including their dependency on zinc ions for catalytic activity (4). The major cell types in human periodontal tissues, including neutrophils, macrophages, epithelial cells and fibroblasts, produce MMPs. The continuous, high secretion of MMPs (such as MMP-2 and MMP-9) by host cells following stimulation by periodontal pathogens contributes to periodontal destruction (5). Therefore, inhibition of MMP production and activity in patients with periodontitis may contribute to reduced periodontal tissue destruction.

Cyclooxygenase (COX) is the first and a critical enzyme involved in the metabolism of arachidonic acid. COX acts on arachidonic acid to form prostaglandins (PGs). Two forms of COX have been characterized: COX-1 and COX-2. COX-1 is responsible for constitutive production of PGs under physiological conditions in various tissues. PGs produced by COX-1 are tissue protective and serve to maintain normal function. Cytokines, growth factors and LPS induce COX-2. Previous studies have demonstrated that PGs produced by inducible COX-2 are involved in the pathogenesis of periodontitis (6,7).

Various signaling pathways, including those mediated by MAPKs, have been reported to be involved in the expression of COX-2 and MMPs (8,9). A major role of MAPKs is the transmission of extracellular signals to the nucleus, where the transcription of specific genes is induced by the phosphorylation and activation of transcription factors. To date, several distinct MAPKs have been identified in vertebrates, and these include ERK1/2, p38 and JNK (11,12).

Fibroblasts are the main constituents of gingival connective tissue. They produce structural connective-tissue proteins, glycoproteins and glycosaminoglycans, as well as immunoregulatory cytokines and chemical mediators that may promote chronic inflammation. Human gingival fibroblasts (HGFs) produce inflammatory cytokines, PGs and MMPs when treated with LPS (13–15). Therefore, HGFs play an important role in periodontal tissue destruction. Regulation of fibroblast inflammatory reactions has been suggested to be one way of preventing and controlling the progression of periodontitis.

Considering the induction of COX-2 and MMPs by LPS, a number of strategies have been designed to block their expression, including the use of different drugs. Among these are nonsteroidal anti-inflammatory drugs (NSAIDs), which produce an improvement in disease management (16).

Zingiber cassumunar has been used in Thai traditional medicine, especially in the treatment of inflammation. Five identified compounds obtained from a hexane extract of the rhizome of *Z. cassumunar* were found to exhibit topical anti-inflammatory activity when tested in the model of 12-*O*-tetradecanoylphorbol-13-acetate-induced ear edema in rats. These anti-inflammatory compounds are phenylbutanoids and cyclohexene derivatives. The active compounds were identified as (E)-4-(3′-4′-dimethoxyphenyl) but-3-en-l-ol, (E)-4-(3′,4′-dimethoxyphenyl) but-3-enyl acetate, cis-3- (2′,4′,5′-trimethoxyphenyl)-4-[(E)-2‴,4‴,5‴-trimethoxystyryl] cyclohex-l-ene, cis-3-(3′,4′-dimethoxyphenyl)-4-[(E)-2‴,4‴,5‴-trimethoxystyryl] cyclohex-1-ene and cis-3-(3′,4′-dimethoxyphenyl)-4-[(E)-3,‴,4‴-dimethoxystyryl] cyclohex-l-ene (17). The essential oil of the rhizome of *Z. cassumunar* was found to exhibit a topical anti-inflammatory effect in animal studies (18). Considering these reports, we hypothesize that *Z. cassumunar* may possess the ability to suppress gingival inflammation in periodontal diseases. We regard the cell-culture system, in which HGFs were treated with LPS, as a suitable *in-vitro* periodontal-disease model. We investigated the effect of *Z. cassumunar* extracts on COX-2 and MMPs with special regard to MMP-2 in LPS-treated HGFs. The effect of this plant extract on MAPK expression by HGFs stimulated with LPS was also investigated in the study. The activation of ERK1/2, p38 and JNK were used as a hallmark of LPS-induced signal transduction in HGFs.

Material and methods

Reagents

Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Monoclonal mouse anti-human vimentin Ig (clone V9), mouse IgG isotype, biotinylated secondary antibody and streptavidin–biotin–horseradish peroxidase conjugate were purchased from Dako (Glostrup, Denmark). COX-1, COX-2, phosphorylated ERK, ERK, phosphorylated p38, p38, phosphorylated JNK, JNK, β-actin monoclonal antibodies and the peroxidase-conjugated secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Gelatin was obtained from Bio-Rad Laboratories (Hercules, CA, USA). MTT [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide], *Escherichia coli* LPS and all other chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

Plant materials and extraction

Rhizomes of *Z. cassumunar* Roxb. were purchased from a local herbal store in Bangkok, Thailand, and their identity was confirmed at the Medici-

nal Plant Research Institute, Department of Medical Sciences, Ministry of Public Health, Thailand. A specimen sample was preserved in our laboratory for future reference. The rhizomes of *Z. cassumunar* were dried and pulverized. Dried, ground rhizomes of *Z. cassumunar* (60 g) were exhaustively extracted with 500 mL of 95% ethanol in a Soxhlet apparatus (Buchi Laboratory, Flawil, Switzerland) for approximately 8 h. The resulting ethanol extract was filtered through Whatman paper No. 1 and concentrated under reduced pressure at 50°C using the Buchi Rotavapor R-200 (Buchi Labortechnik AG, Flawil, Switzerland) to provide *Z. cassumunar* extract (5.5 g).

Preliminary chemical identification of the *Z. cassumunar* extract was performed using a thin-layer chromatography (TLC) technique, as described in the Thai Herbal Pharmacopoeia (19). Briefly, the chromatographic analysis was carried out using a silica gel GF 254 plate (15 cm × 2 cm; Merck, Darmstadt, Germany). A mixture of hexane/ethyl acetate (70 : 30, volume by volume) was used as a mobile phase. To visualize the resolved compounds, the TLC plates were exposed to fluorescent indicator ultraviolet rays (λ = 254 nm) and then sprayed with *p*-anisuldehyde-sulfuric acid reagent, followed by heating at 110°C for 10 min. The respective hR_f (mobility relative to front) values of each resolved compound were calculated by applying the following formula:

hR_f = (Distance traveled by the resolved compound divided by the distance traveled by the solvent) × 100

For sample preparation, a 1000 μg/mL stock solution of *Z. cassumunar* extracts was prepared in distilled water. The extract was filtered and further diluted with the culture medium to the indicated concentration before use. The final concentration of ethanol in the culture medium was $\leq$ 0.01%. At this concentration, the solvent alone showed no cytotoxic effects. Blank controls and solvent controls were also included to check that the ethanol had no detectable effects at the concentration used.

Cell culture

HGFs were prepared from the explants of healthy gingival tissues of patients who underwent minor oral surgery at Thammasat University Hospital. The protocol for human tissue acquisition was in accordance with the requirements issued by the Ethics Commission of Thammasat University. The explants were cut into pieces and cultured in 100-mm-diameter tissue-culture dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and the medium was changed every 3 d for about 2 wk until confluent cell monolayers were formed. After two to three subculture steps performed by trypsinization, populations of homogeneous, slim, spindle-shaped cells, growing in characteristic swirls, were obtained. The cells were used as confluent monolayers at subculture levels 4–8. All experiments were performed under serum-free conditions (0.1% bovine serum albumin). Fibroblast purification was confirmed by vimentin-positive immunocytochemical staining.

Immunocytochemistry

HGFs were seeded at 1.5×10^4 cells per well on two-well chamber slides (Nunc, Roskilde, Denmark) and allowed to grow to approximately 50% confluence before being processed for immunostaining. Briefly, cultured cells were fixed with 4% paraformaldehyde for 30 min at room temperature. To irreversibly block endogenous peroxidase, slides were incubated with 0.3% hydrogen peroxide for 30 min at room temperature. The slides were incubated with either monoclonal mouse anti-human vimentin Ig or mouse igG isotype for 16 h at 4°C, After washing, biotinylated secondary antibody was applied for 1 h, followed by streptavidin–biotin–horseradish peroxidase conjugate for 1 h. Immunostaining was visualized using 3-Amino-9-Ethylcarbazole chromogen and the nuclei were counterstained with Meyer's hematoxylin. All slides were evaluated using a light microscope.

Cell viability assay

HGFs were seeded in 96-microwell plates and incubated for 48 h to allow cell attachment. Near-confluent cells were treated with various concentrations of test compounds in fresh culture medium. Controls and sample-treated cells were incubated for 72 h. The relative numbers of viable cell was then determined using the MTT assay (20).

Treatment of cells

LPS stock solution (0.2 mg/mL) was prepared in sterile water. For optimal LPS-induced activation of MAPKs, HGFs grown on six-well culture plates were stimulated with various concentrations of LPS and incubated at 37°C for the indicated time-periods (up to 60 min). At the end of the incubation period, the cells were lysed directly in the wells of the six-well culture plates. Cell lysates were collected by centrifugation and used for the MAPKs assay. To determine the inhibitory effect of *Z. cassumunar*, HGFs were pretreated with the *Z. cassumunar* extract, at a concentration of 100 μg/mL, for 2 h before being exposed to LPS for the indicated period of time. Cells incubated in culture medium without LPS or *Z. cassumunar* extracts were used as controls. G418 (Genecitin®) was used as a positive control for the inhibition of MAPKs. Cells and culture medium supernatants were collected. Cellular proteins and total RNA were immediately prepared. Conditioned media were stored at −80°C until used for MMP zymography assays.

Western blot analysis

Treated HGFs were washed in cold phosphate-buffered saline, lysed with RIPA buffer [1% Nonidet P-40, 0.5% deoxycholic acid sodium salt, 0.1% sodium dodecyl sulfate (SDS) and phosphate-buffered saline (pH 7.4)] supplemented with 10 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride and 1 × proteinase inhibitor (Complete mini EDTA-free; Roche Applied Sci-

ence, Indianapolis, IN, USA). Cell debris was removed by centrifugation at 10,000 ***g*** for 10 min. Protein content was determined using the Bicinchoninic Acid Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA).

Equal masses of whole-cell protein were separated by electrophoresis on a 12% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. Prestained and biotinylated SDS-polyacrylamide gel electrophoresis standards (Bio-Rad Laboratory, Hercules, CA, USA) were used as molecular-weight standards. The membrane was blocked with 5% bovine serum albumin in Tris-buffered saline containing 0.05% Tween 20 (3 h, room temperature) and incubated overnight at 4°C with antibodies against phosphorylated ERK1/2, ERK1/2, phosphorylated JNK, JNK, phosphorylated p38, p38, COX-1, COX-2 and β-actin, at a dilution of 1 : 500, followed by incubation in the same buffer with appropriate horseradish peroxidase-conjugated secondary antibodies (1 : 40,000 dilution; Pierce Biotechnology) for 1 h at room temperature.

Immunoreactive proteins that were resolved to size as a band by gel electrophoresis were detected using enhanced chemiluminescence (Pierce Biotechnology) and exposed in the high-performance chemiluminescence system (GeneGnome; Syngene, Frederick, MD, USA). The image series were captured using exposure times that varied between 2 and 20 min. The intensity of each protein band was analyzed, and background intensities were subtracted. Western blotting of β-actin was used as a loading control.

RNA extraction and semiquantitative RT-PCR

Total cellular RNA was isolated using the Trizol method (Invitrogen). The concentration of RNA was determined by measuring the absorbance values at 260 and 280 nm. Total RNA (1 μg) was reverse-transcribed using the one-step RT-PCR kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The primer sequences for PCR amplification were as follows:

COX1, forward primer, 5′-GAGTCTTTCTCCAACGTGAGC-3′, and reverse primer, 5′-ACCTGGTACTTGAGTTTCCCA-3′, 350 bp; COX-2, forward primer, 5′-GCAGTTGT TCC AGACAAGCA-3′, and reverse primer, 5′-CAGGATACAGCTCCACA GCA-3′; 232bp; MMP-2 forward primer, 5′-CCACGTGACAAGCCCAT GGGGCCCC-3′ and reverse primer, 5′-GCAGCCTAGCCAGTCGGATT TGTG-3′, 480 bp; glyceraldehyde-3-phosphate dehydrogenase, forward primer, 5′-ACGCATTTGGTCGTATT GGG-3′ and reverse primer, 5′-TGAT TTTGG AGGGATCTGGC-3′, 231bp. Thermocycling was carried out at 50°C for 30 min and 95°C for 15 min, and then 30 cycles of 95°C (denaturation) for 1 min, 60°C (annealing) for 1 min and 72°C (extension) for 1 min were performed. A final extension step was performed for 10 min at 72°C. The PCR products were size-separated by electrophoresis through a 1.8% agarose gel and then visualized following staining with 0.5 mg/L of ethidium bromide. A densitometer (Syngene) was used to measure the density of the PCR-product bands and normalized by comparison with density signatures from the band corresponding to glyceraldehyde-3-phosphate dehydrogenase amplification.

MMP zymography

MMP-2 activities in the cell-conditioned media were detected by gelatin zymography (21). The conditioned media from negative controls, positive controls (conditioned medium of HT 1080 fibrosarcoma cells) and treatment groups were mixed with standard SDS-gel loading buffers containing 2% SDS without β-mercaptoethanol. Samples were not boiled before loading. Prepared samples were electrophoresed through 10% SDS polyacrylamide gels containing 0.2% gelatin. After electrophoresis, gels were washed twice with 25 mL of 2.5% Triton-X-100 on a gyratory shaker for 30 min at room temperature to remove the SDS. The gels were then incubated in 50 mL of reaction buffer (50 mM Tris, pH 8; 5 mM $CaCl_2$ and 10^{-6} M $ZnCl_2$) at 37°C for 18 h.

The gels were stained with Coomassie Brilliant Blue R-250 and destained with methanol/acetic acid/water (50 : 10 : 40, volume by volume) to visualize degraded gelatin. The molecular masses of protein bands corresponding to the presence of gelatinases were indicated by SDS- polyacrylamide gel electrophoresis standards (Bio-Rad Laboratories). Latent and active forms of MMP-2 were detected as clear zones against the blue background at 72 and 67 kDa, respectively. Relative band densities were analyzed using Gene Tools software (Syngene). Calculated values were normalized with the number of corresponding cells in each culture.

Data analyses

Experiments were performed in triplicate throughout this study. All data are presented as mean ± standard error. A significant difference between control and test groups was examined using one-way analysis of variance, with *post-hoc* evaluations carried out using the Bonferroni post-test (Prism5; GraphPad Software Inc., San Diego, CA, USA). A level of significance was considered at $p \leq 0.05$.

Results

Thin-layer chromatography

The TLC chromatogram of *Z. cassumunar* extracts is shown in Fig. 1. The intensity of spots was comparable, which indicated the presence of some definite constituents in the sample. The TLC chromatogram showed 13 spots which caused quenching of fluorescence at an ultraviolet light wavelength of 254 nm. This suggests that the *Z. cassumunar* extracts contained ultraviolet-active components. Detection with anisaldehyde/sulfuric acid revealed that the separation achieved with TLC was consistent with that reported in Thai Herbal Pharmacopoeia. Five spots (1, 2, 3, 4 and 5), with hR_f values of 17, 27, 35, 47 and 56, corresponded to the hR_f values of (E)-4-(3′-4′-dimethoxyphenyl) but-3-en-l-ol, cis-3-(2′,4′,5′-trimethoxyphenyl)-4-[(E)-2‴,4‴,5‴-trimethoxystyryl] cyclohex-l-ene, cis-3-

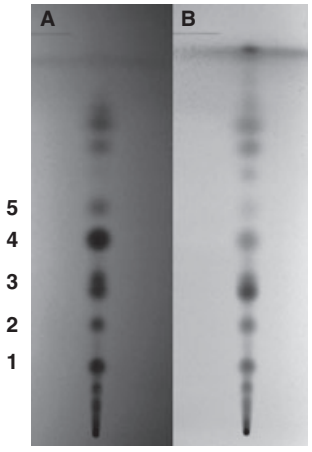

Fig. 1. Thin-layer chomatography (TLC) chromatogram of the ethanol extract of *Zingiber cassumunar*. TLC was carried out using silica gel GF254 as the coating substance and a mixture of hexane/ethyl acetate (70 : 30, volume by volume) as the mobile phase. (A) TLC chromatogram shows spots that cause quenching of fluorescence at an ultraviolet light wavelength of 254 nm. (B). Colored spots on the TLC chromatogram were observed in visible light after spaying with *p*-anisuldehyde-sulfuric acid reagent. Five interesting spots (1, 2, 3, 4 and 5) appear at hRf values of 17, 27, 35, 47 and 56, respectively (for details of these candidate compounds see text).

(3′,4′-dimethoxyphenyl)-4-[(E)-2‴,4‴, 5‴-trimethoxystyryl] cyclohex-1-ene and cis-3-(3′,4′-dimethoxyphenyl)-4-[(E)-3,‴,4‴-dimethoxystyryl] cyclohexl-ene and (E)-4-(3′, 4′-dimethoxyphenyl) but-3-enyl acetate, respectively.

Immunocytochemistry

Cultured cells were identified, based on their morphology, as fibroblasts. Fibroblast purification was confirmed by vimentin-positive immunocytochemical staining (data not shown). These results indicated that the cultured cells used in this study were fibroblasts.

Cytotoxicity

We applied the MTT test to evaluate nontoxic concentrations of *Z. cassumunar* extracts. HGFs were challenged with different concentrations of the extracts. After 72 h of exposure to *Z. cassumunar* extracts at 1.25, 5, 12.5, 25, 125, 500, 1000 and 2000 μg/mL, the amount of cell proliferation was 97 ± 1.18%, 106 ± 1.63%, 100 ± 2.45%, 95 ± 3.37%, 86.42 ± 4.58%, 69.4 ± 3.7%, 18.74 ± 0.97% and 5.62 ± 0.27%, respectively, when compared with untreated control HGFs (100%). No sign of any negative effect was observed after treatment of HGFs with the extracts at concentrations up to 125 μg/mL. Based on the cell-viability results, noncytotoxic concentrations (1.25–125 μg/mL) were chosen for further experiments.

Preliminary experiments showed that 20 μg/mL of *E. coli* LPS was not toxic for HGFs and induced an optimal response (data not shown). This concentration was thus selected to investigate the inhibitory effect of *Z. cassumunar* extracts on the production of MAPKs, COXs and MMPs by HGFs stimulated with *E. coli* LPS. We also examined the combination effects of *Z. cassumunar* extracts and LPS on the proliferation of HGFs. No simultaneous adverse effect on cell viability was found. More than 80% of HGFs survived after cotreatment with LPS (20 μg/mL) and *Z. cassumunar* extracts (100 μg/mL). Treated HGFs did not show any detectable change in morphology compared with untreated cells when examined using phase-contrast microscopy (data not shown).

LPS activates MAPKs in HGFs

To determine whether *Z. cassumunar* extracts modify the phosphorylation patterns of critical proteins by modifying the activity of kinases involved in HGF signal transduction, we treated cells with LPS for different lengths of time. Detection of ERK1/2 phosphorylation occurred 5–30 min after treatment of cells with LPS (20 μg/mL). In all cases, phosphorylation had decreased at 60 min (Fig. 2). Therefore, the effect of LPS on the production of COX-1, COX-2 and MMP-2 by HGFs was investigated at an LPS concentration of 20 μg/mL.

Effects of *Z. cassumunar* extracts on LPS-induced ERK1/2, JNK and p38 phosphorylation in HGFs

Phosphorylation of ERK1/2, JNK and p38 was increased in HGFs treated with LPS alone (Fig. 3). *Z. cassumunar* extracts markedly inhibited phosphorylation of ERK1/2 and JNK in HGFs stimulated with LPS. A lesser inhibitory effect was observed for the phosphorylation of p38. The amounts of ERK1/2, JNK and p38 protein were unaffected by *Z. cassumunar* extracts during the same time interval.

Effects of *Z. cassumunar* extracts on COX-1 and COX-2 expression

Western blot analyses of cell proteins demonstrated that COX-2 proteins were undetectable in inactive cells but appeared in high amounts following incubation with LPS (Fig. 4). The expression of COX-1 protein, in contrast to COX-2 protein, was detected in HGFs without any treatment. However, the expression of COX-1 protein was not enhanced by LPS treatment. The *Z. cassumunar* extract at a concentration of 100 μg/mL displayed a significant down-regulation on the expression of COX-2 protein, but not of COX-1 protein.

Changes in the amounts of COX-2 protein could reflect alterations in either protein synthesis or protein degradation. Therefore, RT-PCR was used to investigate whether *Z. cassumunar* suppressed LPS-mediated induction of COX-2 via a pretranslational mechanism. Our findings indicated that the *Z. cassumunar* extract is a potent inhibitor of the expression of *COX-2* mRNA in LPS-activated HGFs, as measured by a reduced level of mRNAs detected by densitometry scanning (Fig. 5). In contrast, LPS induced no change for the level of *COX-1* mRNAs. The *COX-1* mRNA levels were similar regardless of the concentration of *Z. cassumunar* extracts. These data suggest that the plant extract inhibits the expression of *COX-2* at transcriptional levels.

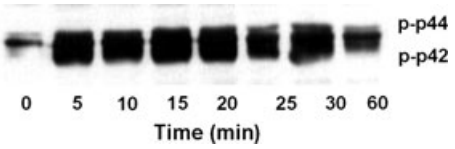

Fig. 2. Lipopolysaccharide (LPS)-induced time-dependent phosphorylation of ERK1/2 in LPS-treated human gingival fibroblasts (HGFs). HGFs were placed in six-well plates then stimulated with LPS (20 μg/mL) for the time periods indicated. Cell lysates were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by western blotting using an antibody against phospho-ERK1/2 (p-p44/42). Data are representative of observations obtained from one of three independent experiments, all of which gave similar results.

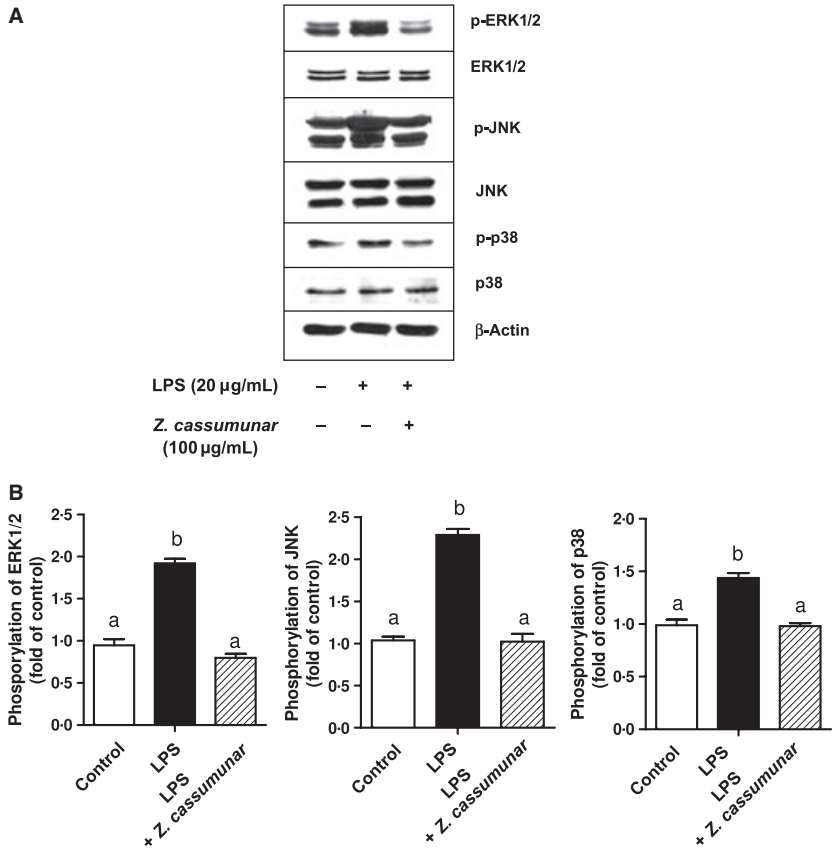

Fig. 3. Effects of *Zingiber cassumunar* on lipopolysaccharide (LPS)-induced activation of MAPKs. (A) The expression of phosphorylated ERK1/2 (p-ERK1/2), ERK1/2, phosphorylated JNK (p-JNK), JNK, phosphorylated p-38 (p-p38) and p38 were analyzed by western blotting. Human gingival fibroblasts (HGFs) were pretreated with the ethanolic extract of *Z. cassumunar* (100 μg/mL) for 2 h and stimulated with LPS (20 μg/mL) for 15 min. Beta-actin was used as a 'loading' control. (B) The expression levels of p-ERK1/2, ERK1/2, p-JNK, JNK, p-p38 and p38 were digitized using Syngene software. Band intensities for phosphorylated proteins were normalized to the corresponding band intensities for total proteins. Data from three independent experiments were averaged and the results are presented as mean ± standard error. Control values (lane 1) were arbitrarily set to 1.0 and the other values were expressed as fold-change. Nonidentical superscript letters represent statistical differences ($p \leq 0.05$).

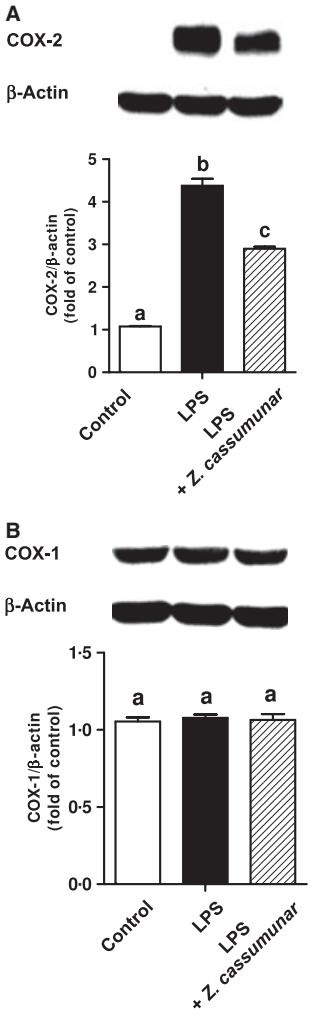

Fig. 4. Effects of treating human gingival fibroblasts (HGFs) with *Zingiber cassumunar* on the expression of cyclooxygenase (COX)-2 (A) and COX- 1 (B) proteins induced by lipopolysaccharide (LPS) (20 μg/mL). HGFs were treated with the ethanolic extract of *Z. cassumunar* (100 μg/mL) for 2 h before stimulation with LPS. Untreated cells were used as a control. After 72 h, cell lysates were collected and subjected to Western blot analysis, as described in the Material and methods. The intensity of each protein band was normalized against β-actin and expressed as a fold of the control. Data are presented as mean ± standard error (SE). Nonidentical superscript letters represent statistical differences ($p \leq 0.05$). All results are representative of three independent experiments.

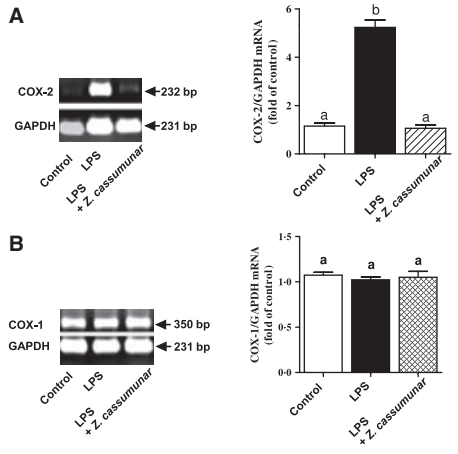

Fig. 5. Effects of treating human gingival fibroblasts (HGFs) with *Zingiber cassumunar* on the expression of cyclooxygenase *COX-2* (A) and *COX-1* (B) mRNAs, induced by stimulation with lipopolysaccharide (LPS) (20 μg/mL). HGFs were treated with the ethanolic extract of *Z. cassumunar* (100 μg/mL) for 2 h before stimulation with LPS. Untreated cells were used as a control. After 8 h, the cells were lysed and total RNA was prepared as described in the Material and methods. The analysis of mRNA was performed by RT-PCR. PCR products were resolved electrophoretically on a 1.8% agarose gel and visualized by staining with ethidium bromide. The staining intensity of each band, indicating the gene-expression level, was normalized against that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and expressed as a fold of the control. Data shown represent the mean value ± standard error. Nonidentical superscript letters represent statistical differences ($p \leq 0.05$). All results are representative of three independent experiments.

Z. cassumunar extracts inhibit MMP-2 expression in LPS-primed HGFs

HGFs were assayed for MMP-2 expression by gelatin zymography. It can be seen from Fig. 6A that gelatinases are secreted by unstimulated HGFs and that these proteins migrated as 72-kDa and 67-kDa protein bands, representing pro-MMP-2 and active MMP-2, respectively. In the presence of LPS, both the latent and active forms of MMP-2 in the culture medium increased significantly after 72 h of incubation. *Z. cassumunar* extracts (100 μg/mL) significantly decreased the activities of latent and active forms of MMP-2 in HGFs ($p \leq 0.05$).

To evaluate the effect of *Z. cassumunar* extracts on MMP-2 regulation, we examined the effect of this plant extract on the levels of expression of *MMP-2* mRNA. We found that LPS stimulated expression of the *MMP-2* gene, resulting in the accumulation of mRNAs (Fig. 6B). Treatment of the HGFs with *Z. cassumunar* extracts (100 μg/mL) resulted in diminishing the *MMP-2* mRNA up-regulation induced by LPS.

Discussion

We used commercially available LPS from *E. coli* to provoke the inflammatory reaction in this study. Basically, *E. coli* is not a periodontal pathogen; however, it has been used in several *in-vivo* and *in-vitro* investigations that examined the periodontal cellular response to LPS (22–24). In addition, it is evident that LPS from *E. coli, Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* share certain biological activities on the host-cell response (25–27).

HGFs produced inflammatory cytokines, such as interleukin (IL)-6 and IL-8, and eicosanoids, such as prostaglandin E_2 (PGE_2), when treated with LPS (28–30). Therefore, we regard this experimental system, in which HGFs were treated with LPS, as *in-vitro* model for periodontal diseases. In our experiments, LPS was used as the prototypical inflammatory stimulus because of its ability to initiate a range of proinflammatory mediators (31). LPS is an effective activator of nuclear factor-kappaB, which is known to play a critical role in the regulation of cell-survival genes and to induce the expression of inflammatory enzymes and cytokines, such as COX-2, inducible nitric oxide synthase, tumor necrosis factor alpha, IL-1β and IL-6 (32–34).

In the current study, 20 μg/mL of *E. coli* LPS was used to challenge the cells. This concentration was relatively high; however, no effect on cell proliferation and cell morphology was observed at the concentration used. Other studies have used a range of 1–100 μg/mL of LPS, which is believed to be within a range of LPS relevant to periodontal inflammation (35, 36). In this study, HGFs were pretreated with the *Z. cassumunar* extracts before stimulation with LPS. It may be argued that a different response may occur during treatment of cells with LPS and the test compound simultaneously. However, the experimental protocol carried out in this study has also been reported in studies of HGFs (37) and human airway epithelial cells (38).

COX-2 is the main enzyme regulating PG synthesis in response to several factors, such as IL-1β, LPS and platelet-derived growth factor (39–41). In HGFs, augmentation of PGE_2 synthesis and release has been considered to be a prominent feature of inflammatory reactions (42) because elevated levels of PGE_2 are detected in inflamed gingival tissues and crevicular fluid, especially from periodontal sites exhibiting attachment loss (43).

Z. cassumunar has previously been shown to possess anti-inflammatory activities *in vitro* and *in vivo* (18, 44). In the present study, we examined the anti-inflammatory effects of an extract of the plant in an *in-vitro* periodontal-disease model. We showed that *Z. cassumunar* extract suppressed LPS-induced COX-2 expression by HGFs. A previous study demonstrated that administration of NSAIDs could also prevent gingival inflammation (16). Clinical studies indicated that the concentration of PGE_2 in gingival crevicular fluid is

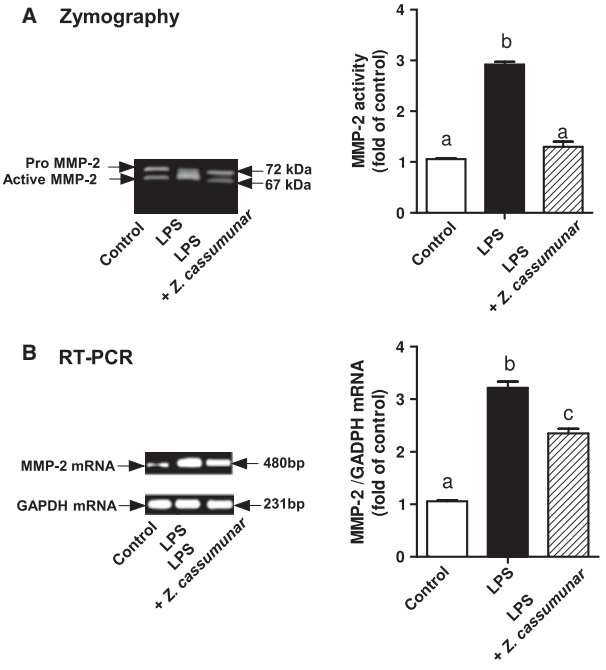

Fig. 6. Effects of *Zingiber cassumunar* on lipopolysaccharide (LPS)-stimulated matrix metalloproteinase (MMP)-2 activities (A) and *MMP-2* mRNA expression (B) in human gingival fibroblasts (HGFs). HGFs were pretreated with the ethanolic extract of *Z. cassumunar* (100 microgram/mL) for 2 h before stimulation with LPS (20 μg/mL). Untreated cell were used as a control. Conditioned media were collected after 48 h, normalized to the cell number, and aliquots corresponding to equal amounts of cells were subjected to gelatin zymography, as described in the Material and methods. Total RNAs were prepared from cell pellets. The expression of *MMP-2* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNAs was analyzed using RT-PCR. Band intensities for *MMP-2* mRNA were normalized to the corresponding band intensities for *GAPDH*. The mean value and standard error of three independent experiments were determined. Control values were arbitrarily set to 1.0 and other values were expressed as fold-change. Nonidentical superscript letters represent statistical differences ($p \leq 0.05$).

increased in periodontal diseases (43) and that its level is decreased by the administration of NSAIDs (45, 46). Considering that both NSAIDs and *Z. cassumunar* extracts suppressed COX-2 expression, this plant may be useful for the treatment of gingival inflammation in periodontal diseases.

There are a number of traditional herbal remedies for the treatment and management of periodontal diseases (47). *Z. cassumunar* extracts may be used in the form of a mouthwash. Recently, the use of sustained-release formulations to deliver drugs to the periodontal pocket has gained interest (48). Therefore, the *Z. cassumunar* extract may be included in a biodegradable gel or film capable of delivering therapeutic concentrations of anti-inflammatory compounds for a prolonged period of time for the adjunctive treatment of periodontitis. Importantly, *Z. casumunar* extract inhibits only COX-2 activity, suggesting that *Z. cassumunar* may exhibit minimal adverse gastrointestinal dysfunction (49). Therefore, based on our data, we project that *Z. cassumunar* may produce anti-inflammatory effects in cells associated with periodontal diseases, such as gingival fibroblasts, as well as exerting beneficial effects on target cells involved with other inflammatory diseases. Taken together, our findings demonstrated that the cellular mechanism of anti-inflammatory action of *Z. cassumunar* extract was, at least in part, a result of COX-2 inhibition.

A critical outcome of periodontal diseases is degradation of the extracellular matrix proteins synthesized by cells from periodontal tissues, and several members of the MMP family, including MMP-1, MMP-2, MMP-8, MMP-9, MMP13 and MMP-14, have been shown to be involved in periodontal tissue destruction (50, 51). In this study, the increase in MMP-2 activities by LPS-induced HGFs supports this role. In further consideration of the mechanism(s) between inflammation of the periodontal tissues (e.g. periodontitis) and MMPs, it has been suggested that MMPs are involved in proteinase-activated receptor-induced periodontitis (52). LPS may stimulate the expression of MMP-2 via PGE_2 production in HGFs (53).

In the present study, *Z. cassumunar* extracts inhibited LPS-induced COX-2 and MMP-2 activation. However, the mechanism(s) by which the plant extracts regulate COX-2 and MMP-2 are currently unknown. Because ERK, JNK and p38 MAPK have been shown to be involved in the LPS-mediated induction of COX-2 and MMP-2 in HGFs (14, 54), we investigated the effects of *Z. cassumunar* extract on the activation of these MAPKs in LPS-stimulated HGFs. Our results demonstrate that *Z. cassumunar* strongly blocked the expression of ERK1/2 and JNK phosphorylation, whereas it had a lesser inhibitory effect on p38 phosphorylation. Therefore, it is likely that *Z. cassumunar* suppressed COX-2 and MMP-2 expression in response to LPS in HGFs via the down-regulation of MAPK phosphorylation (55).

The nature of active compounds in *Z. cassumunar* extracts was not investigated in the study. However, TLC chromatography suggests the presence of some phenylbutanoids and cyclohexane derivatives in our samples. Therefore, it is possible that these compounds play important roles in the inhibition of COX-2 and MMP-2 expression in LPS-treated HGFs.

Traditional medicines that modulate host inflammatory mediators have shown promise for periodontal disease management, and might be beneficial in individuals with an increased risk of periodontal diseases (10). Our results provide a framework for the development of therapies

directed at modulating the host response and for the creation of adjunctive treatments of periodontal diseases by use of components of *Z. cassumunar*.

In summary, this study provides mechanistic insight into the pathways for the anti-inflammatory activity of the *Z. cassumunar* extract. Our results indicate that *Z. cassumunar* extracts inhibit COX-2 and MMP-2 production by LPS-activated HGFs through blocking the proinflammatory signaling pathway involving ERK1/2, JNK and p38. However, further studies are needed to corroborate this mechanism and to isolate an active component(s) in the extract.

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