Inhibitory Effect of *Morus Alba* Stem Extract on Bacterial Biofilms and Matrix Metalloproteinase (MMP)-1, and MMP-2 Expression in Vitro

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Abstract

The objective of this study was to evaluate the inhibitory effect of Morus alba stem extract (MSE) on bacterial biofilms and the expression of MMP-1 and MMP-2 in *Porphyromonas gingivalis* Lipopolysaccharide (LPS)-stimulated periodontal ligament fibroblasts (PDLFs).

Aggregatibacter actinomycetemcomitans and *Pseudomonas aeruginosa* biofilms were formed in flat-bottomed 96-well microtiter plates. The metabolic activity of bacterial cells within biofilms was quantified using the XTT assay. MMP-1 and MMP-2 mRNA and protein expression of MSE-treated PDLFs were evaluated using real-time polymerase chain reaction and enzyme-linked immunosorbent assay, respectively.

MSE showed a significant anti-biofilm effect on both preformed biofilms and biofilm formation of *A. actinomycetemcomitans* and *P. Aeruginosa*. We also found that MSE downregulated MMP-1 and MMP-2 gene expression and reduced the secreted protein level of MMP-1 and MMP-2 stimulated by P. gingivalis LPS. MSE has shown an inhibitory effect on *A. actinomycetemcomitans* and *P. Aeruginosa* biofilms. Moreover, the extract was able to reduce the gene expression and production of MMP-1 and MMP-2. The present observations suggest that MSE is a potential therapeutic agent for the treatment of periodontitis.

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Introduction

Periodontitis is an inflammatory disease initiated by specific bacterial species in the dental plaque biofilm, leading to periodontal tissue destruction, tooth mobility, and finally, tooth loss. The initial etiological factor for the development of periodontal disease is dental biofilm in association with various bacterial species. Periodontal pathogens adhere to and colonize within the extracellular matrix (ECM) in the form of a subgingival biofilm[1] and the net effect of this bacterial biofilm community is to maintain a persistent chronic infection as well as to present resistance to many antibiotics[2]. Previous reports have shown that the presence of gram-negative facultative anaerobes, including *A. actinomycetemcomitans* and *P. aeruginosa* in subgingival sites is associated with periodontitis[3,4].

A. actinomycetemcomitans, an important periodontal pathogen, is known for its strong virulence characteristics that cause periodontal disease. The presence of *A. actinomycetemcomitans* can be indicative of future disease progression[5] and reduced levels of *A. actinomycetemcomitans* after periodontal treatment are consistent with an improvement of periodontal condition[6]. *P. aeruginosa* possesses several factors, such as a regulatory protein (PvrR)[7], and type III secretion system and quorum sensing (QS)-regulated factors[8], that contribute to persistent colonization by different strains and thus they become hard to eradicate. A study using the DNA probe technique reported that the association between *A. actinomycetemcomitans* and *P. aeruginosa* in the subgingival biofilm increased significantly the likelihood of a subject having aggressive periodontitis[9].

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MMPs are involved in the etiology of many inflammatory diseases, including periodontal diseases. Gingival tissue staining from subjects with periodontal inflammation revealed large quantities of metalloprotease-1 (MMP-1) and metalloprotease-2 (MMP-2)[10]. The levels of MMP-1 and MMP-2 in gingival crevicular fluid and saliva were higher in periodontitis lesions than normal periodontal tissue[11]. Furthermore, the expression of MMP-1 and MMP-2 were upregulated in human periodontal ligament cells stimulated with interleukin-1 beta[12].

Morus alba L., also known as mulberry has long been used in traditional medicine for several ailments. The ethanolic extract of *M. alba* stems contained a high amount of oxyresveratrol(trans-2,3`,4,5`-tetrahydroxystilbene) and demonstrated anti-inflammatory activities through the inhibition of iNOS, NO, and COX-2 production[13]. It has been reported that oxyresveratrol from *M. alba* possesses anti-inflammatory activity in LPS-stimulated RAW 264.7 macrophage cells and reduced paw edema in rats[14]. In addition, *M. alba* stem extract (MSE) can inhibit the growth *of* periodontopathic bacteria and the expression of IL-6 and IL-8 in *P. gingivalis* LPS-stimulated human PDLFs[15]. However, the effect of MSE on anti-biofilm formation and MMPs expression in PDLFs have never been investigated. To provide information on the use of MSE as an *adjunctive* agent in periodontal therapy, we determined the effect of MSE on *A. actinomycetemcomitans* and *P. aeruginosa* biofilms formed and the expression of MMP-1 and MMP-2 in *P. gingivalis* LPS-stimulated PDLFs in vitro.

Materials and methods

Plant materials and preparation of plant extract.

M. Alba stems were obtained from the Queen Sirikit Sericulture Center, Tak Province, Thailand. The fresh stems were chopped and dried. Then, the dried plant was extracted by maceration technique using 80% ethanol for 2 cycles at room temperature as previously described[15]. MSE was dissolved in 0.05% in dimethyl sulfoxide (DMSO) to give a concentration of 100 mg/ml as a stock solution and diluted with the culture medium to obtain the designed concentrations.

Bacteria and culture conditions

A. actinomycetemcomitans strain ATCC 29523 was obtained from the American Type Culture Collection, Manassas, VA, USA. and *P. aeruginosa* strain ATCC 27853 was obtained from microbiology laboratory of the Department of Medical Sciences, Ministry of Public Health, Thailand. *A. actinomycetemcomitans* was grown at 37°C under an atmosphere of 5% carbon dioxide. Culture media was Brain Heart Infusion (BHI) broth /agar. *P. aeruginosa* was grown at 37°C under aerobic conditions. Culture media was Tryptic Soy Broth (TSB) broth/agar. Tryptic soy broth supplemented with 0.2 % glucose (Merck KGaA, Darmstadt, Germany) was used to form biofilm in microtiter plates. The concentration of bacteria was determined with a spectrophotometer at an optical density of 625 nm (OD 0.08-0.1 = 1-2x10^8 cells/ml).

Inhibitory effects of MSE against *A. actinomycetemcomitans* and *P. aeruginosa* on biofilm formation.

To determine the effects of MSE on the formation of biofilms by these periopathogenic bacteria, the *A. actinomycetemcomitans* and *P. aeruginosa* biofilm formation in wells of commercially available presterilized, flat-bottomed 96-well polystyrene microtiter plates (Thermo Fisher Scientific, Denmark). The MSE stock solution was dissolved in dimethyl sulfoxide (Sigma, St. Louis, MO, USA) to give a concentration of 300 mg/ml. From this stock, the solution was diluted to 0.5-8 mg/ml at a final concentration with Brain Heart Infusion broth for *A. actinomycetemcomitans* and Tryptic Soy Broth for *P. aeruginosa* in a microtiter plate. An equal volume of the *P. aeruginosa* or *A. actinomycetemcomitans* suspension (10^6 cells/ml) was added and mixed with the MSE. A bacterial suspension with 2.66 % DMSO was used as a negative control whereas 0.2 % chlorhexidine was used as a positive control. The plates were placed in an incubator at 37 ºC under an atmosphere of 5% carbon dioxide for biofilms of *A. actinomycetemcomitans* were cultured for 24 h and *P. aeruginosa* biofilms were cultured for 24 h in 37 ºC in aerobic condition. After biofilm formation, the medium was aspirated, and non-adherent cells were removed by thoroughly washing the biofilms three times with sterile phosphate-buffered saline (PBS). A series of MSE-free wells and biofilm-free wells was also included to serve as controls. The effect of each concentration of MSE on the inhibition of

biofilm formation was determined by using an XTT reduction assay described below. Percentage of biofilm formation of each agent was calculated using the formula [(OD490sample/OD490control)] X 100 % . All experiments were repeated on three separate occasions, with triplicate determinations on each occasion.

Antibacterial activities of MSE against preformed *A. actinomycetemcomitans* and *P. aeruginosa* biofilms**.** *A. actinomycetemcomitans* and *P. aeruginosa* biofilms were formed by pipetting of standard cell suspension of *A. actinomycetemcomitans* or *P. aeruginosa* (100 µl) into the wells of flat-bottomed 96-well polystyrene microtiter plates and covered with its original lid. The *plates were incubated* for 24 h *using* the *same conditions* described for bacterial *biofilm formation*. After the incubation time, the medium was discarded and non-adherent cells were removed by thoroughly washing the biofilms three times in sterile PBS. The MSE was then added to the biofilms in serially double diluted concentrations and incubated for a further 24 h. A series of agent-free wells and biofilm-free wells were also included to serve as controls. The effects of each concentration of MSE on preformed biofilms were determined by using XTT reduction assay. Percentage of biofilm formation of each agent was calculated. All experiments were repeated on three separate occasions.

XTT reduction assay.

A. actinomycetemcomitans and *P. aeruginosa* cell viability were determined using a colorimetric XTT reduction assay that measures the activity of mitochondrial dehydrogenase. XTT solution (1 mg/ml) was prepared by dissolving XTT powder in PBS and the solution was filter-sterilized (0.22-lm pore size filter). XTT solution was mixed with freshly prepared menadione solution (0.4 mM) at 20:1 (v/v) immediately prior to the assay. XTT-menadione solution was transferred to each well containing prewashed biofilms and incubated in the dark for 2 h at 37ºC. After the incubation, the colored supernatant (100 µl) was transferred to new microtiter plates and the optical density of the supernatant was measured at 490 nm with a microtiter plate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Preparation and culture of human periodontal ligament fibroblast cells.

This study was approved by the Naresuan *University Institutional Review Board* (COE No.324/2019). Human PDLFs were harvested from caries-free and periodontally healthy teeth extracted for orthodontic reasons with informed consent. The periodontal ligament tissue explants were plated in 35-mm tissue culture dishes and grown in Dulbecco's Modified Eagle's medium (DMEM: Hyclone™, South Logan, UT, USA) with 10% FBS (Hyclone™), 2 mM L-glutamine (Hyclone™), 100 U/mL penicillin (Hyclone™) and 100 µg/mL streptomycin (Hyclone™) at 37ºC, 5% CO_2, and 95% humidity. After reaching confluence, the cells were detached with 0.25% Trypsin-EDTA (Hyclone™). Cells between the 3rd and 5th passage are used in the following studies.

Assessment of MMP-1 and MMP-2 mRNA expression.

PDLFs were seeded in a 6-well plate at a density of $5x10^5$ cells per well. Following 1 h of pre-incubation with nontoxic concentrations of MSE (1.25, 2.5, 5.0 µg/ml), cells were treated with of 10 µg/ml *P. gingivalis* LPS for 24 h. Total RNA was then isolated by Nucleospin RNAII (Macherey-Nagel GmbH&Co. KG) according to the manufacturer's intructions. cDNA was synthesized from 1 µg of total mRNA and mixed with LightCycler 480 DNA SYBR Green I Master (Roche). Two-step quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was performed in duplicate using the LightCycler 480 II Real-Time PCR System (Roche) Two-step quantitative RT-PCR was performed in duplicate using the LightCycler 480 II RT-PCR System (Roche Diagnostics) as previously described[16]. The sequences of the PCR primers were used as follows: MMP-1, forward, GATGGGAGGCAAGTTGAAAA, and reverse, CTGGTTGAAAAGCATGAGCA; MMP-2, forward, ATCCGTGGTGAGATCTTCTTCTT, and reverse, AGCCAGGATCCATTTTCT TCTT; and GAPDH, forward, GAAGGTGAAGGTCGGAGTC, and reverse, GAAGATGGT GATGGGATTTC. Data were analyzed with the LightCycler® 480 software version 1.5. Quantification was calculated using the starting number of fluorescence units of the cDNA of interest relative to that of GAPDH cDNA in the same sample. All measurements were performed in duplicate and are repeated at three different occasions.

Analysis of MMP-1 and MMP-2 protein expression.

After PDLFs were treated with MSE and *P. gingivalis* LPS, the supernatants were collected and the levels of MMP-1 and MMP-2 protein were measured using the human MMP-1, and MMP-2 enzyme-linked immunosorbent assay (ELISA) kits (Abcam, Cambridge, MA, USA) according to the manufacturer' s instructions. Samples were added to the MMP-1 and MMP-2 monoclonal antibody coated well plate, and MMP-1 and MMP-2 bound to the immobilized capture antibody. After washing, a biotinylated anti-human MMP-1 and MMP-2 detection antibody was added. 1 0 0 µl Avidin-horseradish peroxidase was subsequently added, followed by 1 0 0 µl TMB Substrate Solution. The optical densities of each well at 450 nm were determined using a microplate reader (BIO-RAD Laboratories, Hercules, California, USA) . All measurements were performed in duplicate and are repeated at three different occasions.

Statistical Analysis

Statistical analyses were performed using the SPSS 22.0 software. All data were expressed as mean ± standard deviation (SD). Differences between experimental groups were analyzed by ANOVA followed by Tukey HSD comparison test. The level of statistical significance was set at $P < 0.05$.

Results

The inhibitory effect of MSE on *A. actinomycetemcomitans* and *P. Aeruginosa* biofilms was assessed using the XTT reduction assay. Our results showed that MSE was active against *A. actinomycetemcomitans* and *P. Aeruginosa* biofilm formation and preformed biofilms in a time-dependent manner. MSE induced significant inhibition of biofilm formation against *A. actinomycetemcomitans* at the concentration of 8 mg/ml ($p<0.05$) and at the concentration of 2 mg/ ml against *P. Aeruginosa* compared with the control group ($p<0.05$)(Figure 1).

In addition, the concentration of 8 mg/ ml MSE provided a statistically significant reduction in preformed biofilms for *A. actinomycetemcomitans* ($p<0.05$) and at the concentration of 4 mg/ ml against *P. Aeruginosa* ($p<0.05$)(Figure 2). MSE was less effective on the biofilm formation and performed biofilms of *A. actinomycetemcomitans*.

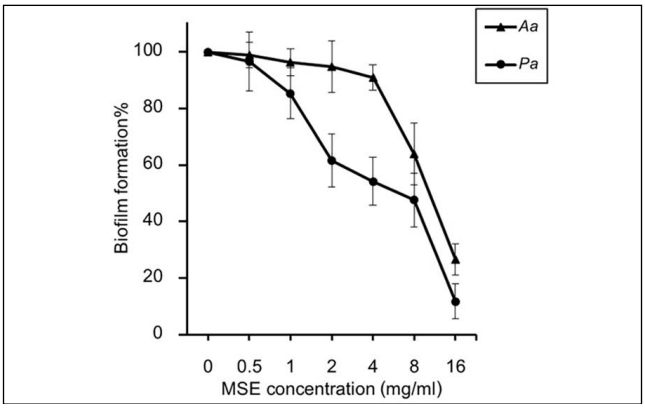

Figure 1. Effect of different concentrations of MSE on bacterial biofilm formation. (Filled triangle) *A. actinomycetemcomitans*, (filled circle) *P. aeruginosa*. Biofilm formation was evaluated by XTT reduction assay, and the results were presented as the percentage compared to the control biofilm formed without MSE treatment. Biofilm formation results represent the mean ± standard deviation.

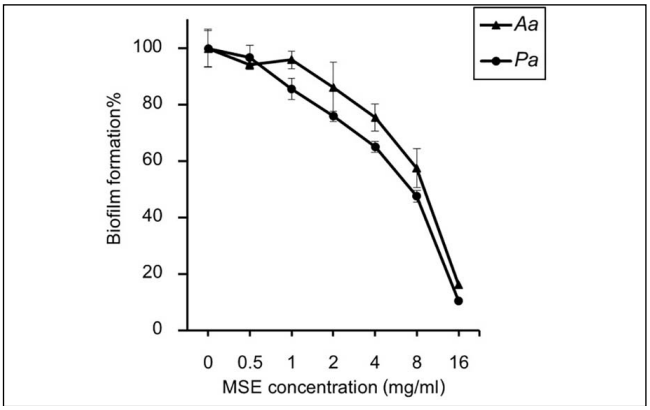

Figure 2. Effect of different concentrations of MSE on preformed bacterial biofilm. (Filled triangle) *A. actinomycetemcomitans*, (filled circle) *P. aeruginosa*. Biofilm formation was evaluated by XTT reduction assay, and the results were presented as the percentage compared to the control biofilm formed without MSE treatment. Biofilm formation results represent the mean ± standard deviation.

The MMP-1 and MMP-2 expressions were decreased after all concentrations of MSE treatment in PDL fibroblasts stimulated with *P. gingivalis* LPS. mRNA expression levels of MMP-1 and MMP-2 were determined by quantitative RT-PCR and protein levels were analyzed by

ELISA. 1.25μg/ml MSE significantly reduced both MMP-1 and MMP-2 mRNA (Figure 3) as well as the protein levels of MMP-1 and MMP-2 (p<0.05)(Figure 4).

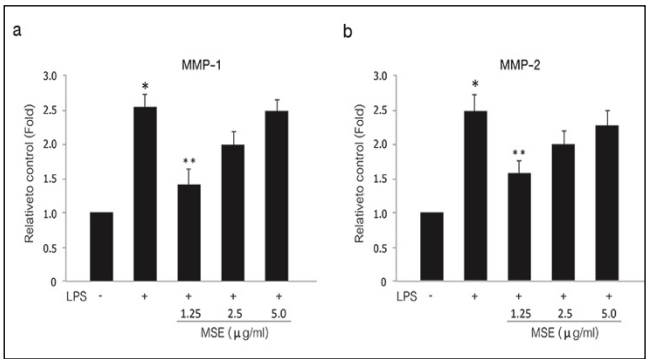

Figure 3. Modulation of *P. gingivalis* LPS induced MMP-1 (A) and MMP-2 (B) mRNA expression by MSE. PDLFs were incubated with 1.25, 2.5 and 5.0 μg/ml MSE or 0.05% DMSO for 1 h, followed by 10 μg/ml *P. gingivalis* LPS stimulation. After 24 h, cells were extracted for Real time PCR. Levels of MMP-1 and MMP-2 mRNA were normalized to GAPDH. mRNA expression results represent the mean ± standard deviation. * indicates significant difference from the non-treated cells; ** indicates significant difference from *P. gingivalis* LPS treatment alone (p < 0.05).

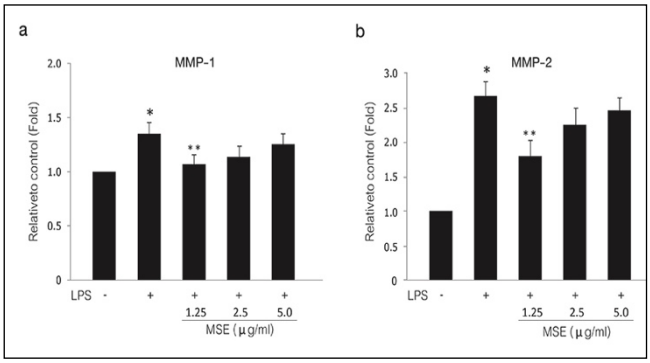

Figure 4. Modulation of *P. gingivalis* LPS induced MMP-1 (A) and MMP-2 (B) protein expression by MSE. PDLFs were incubated with 1.25, 2.5 and 5.0 μg/ml MSE or 0.05% DMSO for 1 h, followed by 10 μg/ml *P. gingivalis* LPS stimulation. The culture supernatants were collected 24 h later. The amount of MMP-1 and MMP-2 protein expression were measured by ELISA. Protein expression results represent the mean ± standard deviation. * indicates significant difference from the non-treated cells; ** indicates significant difference from *P. gingivalis* LPS treatment alone (p < 0.05).

Discussion

In recent years, anti-periodontal pathogen and anti-inflammation activities of medical plant extracts gained higher interest due to easy availability, cost affectivity and nontoxic nature[16-17]. There is evidence that *M. alba* extracts possess several bioactive components, which can significantly target different sites or pathways of bacteria or cells, and hence stopping or inhibiting the growth of pathogens[18] or suppressing the inflammatory response[19,20].

Oxyresveratrol, a compound in MSE was found to be effective against selected Gram-negative periodontal bacteria[21]. These findings are in accordance with our previous report that addressed the effect of MSE on *P. gingivalis* and *A. actinomycetemcomitans* in a planktonic form[15]. However, the etiology of periodontal diseases is associated with colonization and microbial biofilm development. This study demonstrated that MSE had a significant anti-biofilm effect against *A. actinomycetemcomitans* and *P. aeruginosa* under the condition that the biofilms were preformed and mature. Moreover, MSE inhibited the establishing of *A. actinomycetemcomitans* and *P. aeruginosa* biofilms in a concentration-dependent manner and the inhibitory effect against *A. actinomycetemcomitans* required higher concentration of MSE which is regarded as a potentially drug-resistant pathogen. It is evident from previous studies that bacteria inside the biofilm are much more resistant to antimicrobial agents than the preformed state[22]. We found that inhibition of *P. Aeruginosa* preformed biofilm required higher MSE concentrations than inhibition of the formation of biofilm. It is possible that preformed *P. Aeruginosa* biofilm may provide resistance to MSE treatment by limiting the diffusion of substances through the matrix. Previous *studies have* addressed the roles *of* plant-based products in preventing biofilm formation through the following aspects, inhibition of the formation of the polymer matrix, suppression of cell adhesion and attachment, interrupting ECM generation and decreasing virulence factor production, thereby blocking QS network and biofilm development[23].

In addition, biofilm production of six bacterial strains was inhibited when extracts of plants interacted with additional forces like Brownian, Lifshitz-Van der Waals, sedimentation,

and electrostatic interactions, which promote bacterial attachment to surfaces[24]. Although some Chinese herbal medicines inhibit *P. aeruginosa* biofilm formation by suppressing the expression of biofilm-associated genes[25], further studies are required to elucidate the mechanism underlying this phenotypic response.

Inhibition of MMP activity means fighting against connective tissue destruction by preventing collagen degradation, hence stopping the overexpression of host-derived MMPs has an important role in the prevention of periodontitis progression. In the current study, *decreased levels* of both MMP-1 and MMP-2 mRNA and protein in LPS stimulated hPDL fibroblasts were found after MSE treatment.

There are cases where the suppressive effect on the production of proinflammatory mediators did not show simple dose-dependent effects and in this regard, it can be inferred that the MSE showed an optimal concentration for MMP-1 and MMP-2 expression at 1.25 µg/ml. According to our study, the crude *extracts* from *some herbs* have shown significant MMP-1, MMP-8 and MMP-13 inhibitory effect[26]. Flavonoid myricetin, usually found in tea and medicinal plants, decreases the expression of MMP-1 and MMP-8 and the enzyme activity of MMP-2 and MMP-8 in LPS-activated PDLFs[27]. The production of MMP-1 and MMP2 were inhibited when LU105 was added in the culture media of gingival fibroblasts derived from inflamed tissue[28].

In another study, MSE has the same inhibitory effect on MMP-13 production by human chondrocytes as oxyresveratrol at an equivalent amount found in MSE[29]. A previous report showed that oxyresveratrol suppressed NF-kB and MAPK signaling pathways by inhibiting the phosphorylation of IkB-α, NF-kB translocation to the nucleus and phosphorylation of JNK and p38[30]. It is therefore possible that these pathways may be one of the key mechanisms responsible for the MMP-1 and MMP-2 inhibition of MSE. However, studies of the anti-inflammatory activity of MSE on other oral cells and inhibitory effects on the biofilm formation of other periodontal pathogens are necessary to evaluate their possible use for the treatment of periodontal diseases.

Conclusions

The results of the present study demonstrated that MSE possessed in vitro activity in inhibiting *A. actinomycetemcomitans* and *P. aeruginosa* biofilm formation. MSE was able to *decrease* MMP1 and MMP-2 expression in LPS-stimulated hPDLF. Thus, our findings suggest that MSE could be employed as a promising therapeutic agent for treating periodontitis.

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Declaration of Interest

The authors declare no conflicts of interest.

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