

Growth inhibitory effect of mulberry leaf extract on *Streptococcus mutans* in vitro

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뽕잎 추출물의 *Streptococcus mutans*에 대한 성장 억제 효과

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연구목적: 본 연구는 뽕잎(mulberry leaf) 추출물이 치아우식증의 주원인 균인 *Streptococcus mutans*의 성장억제에 미치는 영향을 알아보기 위하여 시행하였다.

연구방법: 추출물의 세균 성장 억제 효과는 평판희석법을 시행하여 추출물의 농도(5, 20, 50 mg/mL)에 따라 시간별(배양 직후, 6, 12, 24시간)로 *Streptococcus mutans*를 배양한 후 생균수를 측정하여 평가하였다. 또한 생약 추출물이 구강 내에서 안전하게 사용되기 위해서는 구강내 정상 연조직 세포에 독성이 없어야 하므로 사람의 정상 치은섬유아세포에 0, 5, 20, 50 mg/mL의 추출물을 처리하여 MTT로 세포독성을 검사하였다.

연구결과: 뽕잎 추출물의 농도에 따른 시간별 생균수 측정 결과, 생균수는 시간의 변화에 따라 통계적으로 유의하게 변화하였으며, 농도에 따라 생균수에 유의한 변화가 있었다($P < 0.05$). 추출물의 각 농도(5, 20, 50 mg/mL)에서 88% 이상의 높은 세균 성장 억제율을 나타내었다. 정상 치은섬유아세포에 뽕잎 추출물을 처리하여 독성 유무를 측정한 결과, 20 mg/mL 이상의 농도에서 세포독성을 나타내었다.

결론: 뽕잎 추출물은 시간의 변화에 따라 생균수를 감소시켰으며, 대조군과 비교하여 높은 성장 억제율을 보여 억제능이 뛰어난 것으로 나타났다. 따라서 뽕잎 추출물은 다양한 우식 예방제제로서 사용될 수 있을 것으로 생각되었다.

Key Words: Antimicrobial, Cytotoxicity, Microbiology, Mulberry leaf, *Streptococcus mutans*

색인: 미생물, 성장 억제 효과, 세포독성, 뽕잎, *Streptococcus mutans*

Introduction

Dental caries, the most common oral disease, is a multifactorial disease caused by interactions among bacteria

within the dental plaque, food, and saliva, resulting in tooth destruction. Mutans streptococci are generally accepted as the main bacteria responsible for dental caries¹. *Streptococcus mutans* (*S. mutans*) has been strongly implicated as

the causative organism of dental caries and it is frequently isolated from human dental plaque²⁾. *S. mutans* produces both water-soluble and water-insoluble glucan from sucrose through the action of glucosyltransferases (GTase)^{3,4)}. Shouji et al¹⁾ and Ikeda et al⁵⁾ have reported on the possibility of preventing dental caries by eliminating the caries-inducing bacterial factors. Katsura et al⁶⁾ described three prime targets for the prevention of dental caries: using antimicrobial agents against *S. mutans*, inhibiting the adhesion of *S. mutans* to the tooth surface, and inhibiting GTase-forming water-insoluble glucan.

Recently, an increasing interest in the effect of natural substances on resident oral microflora has developed and has focused on their ability to promote the growth of beneficial organisms and inhibit the growth and metabolism of species associated with disease, particularly plaque-related diseases such as dental caries⁷⁾. Several studies have demonstrated the antimicrobial activity of natural substances against select oral pathogens. For example, shiitake extracts, green tea extracts, propolis extracts, and oolong tea extracts have been shown to inhibit the growth and adhesion of *S. mutans*^{1,8-11)}.

As part of an ongoing study of natural substances with the potential to effectively prevent dental caries, this study focused on mulberry leaf extract. Mulberry leaves have been shown to produce pharmacological activity, such as antihypertensive, hypocholesterolemic, anti-atherogenesis, and anti-tumor promoting activities as well as anti-diabetic effects¹²⁻¹⁶⁾.

Epicatechin and epigallocatechingallate separated from mulberry leaf were associated with substantial inhibition of the growth of *Clostridium perfringens*¹⁷⁾. However, relatively little attention has been paid to the antimicrobial effect of mulberry leaf extract against dental-plaque formation in the service of the prevention of dental caries. If this substance has an antimicrobial effect on cariogenic bacteria, especially *S. mutans*, it could be a useful agent in oral health products such as mouth rinse and toothpaste. Thus, it is important to determine whether this extract can inhibit the growth of *S. mutans*.

In this study, our hypothesis was that mulberry leaf as a possible anti-cariogenic agent would have a growth inhibitory effect on *S. mutans* and that it would have no cytotoxic effect on human gingival fibroblasts at certain concentrations.

Materials and Methods

1. Extract preparation

Methanolic extract of mulberry leaf was used in this study. The mulberry leaves were pulverized, and 60 g was added to 600 mL of an 85% methanol solution for 3 h. The samples were extracted using an ultrasonic extractor (Sonics-JAC 4020, Jinwoo Co., Korea) for 1 h and filtered through a membrane filter (No. 5A, Advantec, Japan). The same method was repeated three times. The filtered extracts were concentrated using a decompression concentrator (R-210, Buchi, Switzerland), and the concentrated solution was lyophilized (FD 8512, Ilshine Lab, Korea) to obtain a powder. The extract was stored in the dark at 4°C until used.

2. Bacterial strains and culture conditions

S. mutans KCTC 3065 was obtained from the Korea Research Institute of Bioscience and Biotechnology. *S. mutans* was inoculated anaerobically to 4 mL of a brain-heart infusion (BHI; Difco Laboratories, USA) broth containing 10% sucrose, and incubated at 37°C in a chamber with an atmosphere containing 5% CO₂ (Forma Scientific Co., USA) for 48 h.

3. Determination of antimicrobial activity and growth inhibition rate against *S. mutans*

The mulberry leaf extract was dissolved in sterile distilled water and diluted with BHI broth to final concentrations of 5, 20 and 50 mg/mL. A 0.05 mL aliquot of *S. mutans* culture was introduced into fresh BHI broth containing 5, 20, and 50 mg/mL of the extracts in a test tube. The culture was continued in a chamber at 37°C in an atmosphere containing 5% CO₂. To determine the viable cell counts in the broth containing mulberry leaf extract over time (immediately after cultivation and at 6, 12, and 24 h), the culture broth was diluted with BHI to 10⁻⁶, and the diluted solution was introduced into mitis salivarius agar (MS; Difco Laboratories, USA) plates. The plates were incubated at 37°C for 72 h, and the colony-forming units (CFU) were counted. The control was produced by simply introducing the *S. mutans* into fresh BHI broth without the extract. The experiment was repeated three times. The rate of growth inhibition of *S. mutans* associated with the extract was calculated using the following formula¹⁸⁾:

$$\text{Growth inhibition rate (\%)} = \frac{\text{CFU in control} - \text{CFU in treated extract}}{\text{CFU in control}} \times 100$$

4. Tissue culture

For safety purposes, the cytotoxicity of the extract was evaluated on human gingival fibroblasts by MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, sigma, USA] assay. Human gingival fibroblasts were taken from patients who had visited the Department of Periodontology at the Dental Hospital of Chonnam National University, Korea. The human gingival tissue was incubated at 37°C with 5% CO₂, 1% antibiotic-antimycotic (Gibco BRL, Grand Island, NY, USA), and Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL) containing 10% fetal bovine serum (FBS; Gibco BRL). The study protocol was approved by the Chonnam National University Hospital Institutional Review Board (#IRB: I-2008-06-069).

5. MTT cytotoxicity

The fibroblasts were washed with phosphate-buffered saline (PBS; Gibco BRL) and treated with a trypsin-EDTA solution (Gibco BRL). Next, 2×10^4 cells/well were added to 96-well plates and incubated at 37°C in an atmosphere containing 5% CO₂ for 24 h. The mulberry leaf extract was dissolved in sterile distilled water and diluted in media to final concentrations of 0, 5, 20 and 50 mg/mL. Fibroblasts were treated with the mulberry leaf extract for 24 h. The MTT solution (10 μL) was added to each culture plate well for 3 h and then removed. Dimethyl sulfoxide (DMSO; Sigma, USA) (50 μL) was added to each well to dissolve the formazan crystals produced. The optical density of each well was measured using a microplate reader (VERSA max, Molecular Devices, USA) at 490 nm. The results were compared with the 0 mg/mL control. All experiments were repeated three times.

6. Statistical analysis

The experiments were performed in triplicate and the means and standard deviations were calculated. The CFU and growth inhibition rate of *S. mutans* at different concentrations of the herbal extract according to the incubation time were tested by repeated-measures ANOVA. The cytotoxicity of extract concentrations was analyzed using a non-parametric Kruskal-Wallis test among groups, and a Mann-Whitney test was used as a post-hoc test. The Statistical Package for the Social Sciences (version 14.0; SPSS Inc. Chicago, IL., USA) was used for statistical analyses.

Results

1. Antimicrobial activity and growth inhibition rate against *S. mutans*

The growth of *S. mutans* was inhibited by the presence of the mulberry leaf extract (Fig. 1). The number of *S. mutans* cultured in the medium with mulberry leaf extract declined significantly over time ($P < 0.05$), and significant differences ($P < 0.05$) were observed among the 5, 20, and 50 mg/mL concentrations. In contrast, the number of *S. mutans* cultured in the medium without mulberry leaf extract increased rapidly at 6 h and reached a plateau at 12 h. The number of *S. mutans* cultured with 5, 20, and 50 mg/mL of mulberry leaf extract decreased rapidly at 12 h and contained the fewest number of *S. mutans* at 24 h.

The growth rate of *S. mutans* was significantly different ($P < 0.01$) at each concentration as a function of time. All three concentrations of mulberry leaf extract (5, 20, and 50 mg/mL) showed a high growth inhibition rate, >88%, after 6 h as compared with the control (Table 1).

2. Cytotoxicity

The cytotoxicity of the extract to human gingival fibroblasts was examined. The optical density decreased significantly ($P < 0.05$) as a function of increasing concentrations of extract. The viability of the human gingival fibroblasts incubated with 5 mg/mL was significantly higher in the cells that were treated with the mulberry leaf extract than in the control without extracts, but the viability at ≥ 20 mg/mL was significantly lower in the cells that were treated with the mulberry leaf extract than in the control (Table 2).

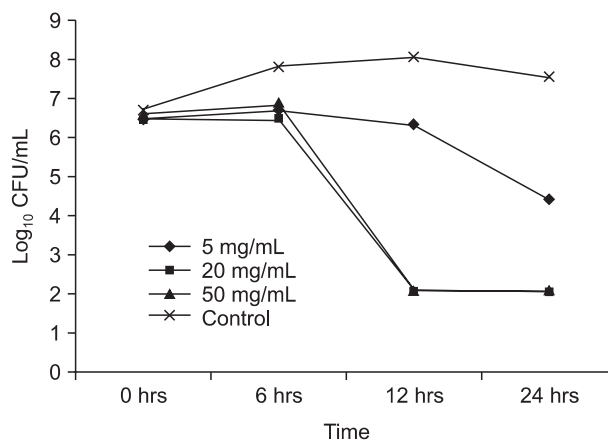


Fig. 1. Inhibitory activity of mulberry leaf extract on *S. mutans*. The CFU of *S. mutans* at different concentrations (0, 5, 20, and 50 mg/mL) of mulberry leaf extract according to the incubation time (immediately after cultivation, 6, 12, and 24 h).

Table 1. Percentage of growth inhibition of *S. mutans* treated with various concentrations of mulberry leaf extract according to incubation time unit: %

Mulberry leaf extract (mg/mL)*	Time (h)			
	0	6	12	24
Control ^a	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
5 ^b	55.56±9.62	91.10±5.78	98.12±0.30	91.05±9.27
20 ^b	48.61±14.63	94.85±2.29	99.26±1.28	98.77±2.14
50 ^b	36.11±12.73	87.54±3.17	99.43±1.00	98.77±2.14

Values represent the mean ± SD from triplicate experiments.

* $P < 0.01$ as per repeated-measures ANOVA.

^{a,b}The same letter indicates no significant difference according to Tukey's test at $\alpha = 0.05$.

^{a,b}The same character indicates no significant difference at 0, 6, 12, and 24 h.

Table 2. Cytotoxicity of mulberry leaf extract on human gingival fibroblasts according to MTT assay

Mulberry leaf extract (mg/mL)	Optical density*
0	0.38±0.04 ^a
5	0.46±0.03 ^a
20	0.22±0.02 ^b
50	0.13±0.01 ^c

Values represent the mean ± SD from triplicate experiments.

* $P < 0.05$, by Kruskal-Wallis test.

^{a,b,c}The same character indicates no significant difference according to the Mann-Whitney test at $\alpha = 0.05$.

Discussion

Dental caries is generated by bacteria in dental plaque and begin when a sufficient number of bacteria are active enough to escape the host's defense¹⁹. Thus, it is believed that dental caries can be prevented by reducing the amount of bacteria in dental plaque. Accordingly, this study examined how an extract of mulberry leaf inhibited the growth of *S. mutans*.

In this study, treatment with 5, 20 and 50 mg/mL of mulberry leaf extract was associated with greater growth inhibition against *S. mutans* over time compared with that associated with the control. However, the differences of growth inhibition rate were no significantly different among the 5, 20 and 50 mg/mL. Because high growth inhibition rate was represented from 5 mg/mL. The 20 and 50 mg/mL extract treatments showed greater growth suppression against *S. mutans* than did the 5 mg/mL treatment, they also displayed cell toxicity. These results demonstrate that mulberry extract can exert an inhibitory effect against *S. mutans*, and that 5 mg/mL used in this study can be considered to be a safe concentration of mulberry leaf extract in terms of growth suppression and low cell toxicity.

Relatively little attention has been paid to the antimicrobial effect of mulberry leaf extract against *S. mutans*. It was difficult to determine the concentration of mulberry leaf extract. Therefore, 5, 20, and 50 mg/mL concentrations was determined based on reported natural substances that have antibacterial effect of oral pathogens.

The most likely reason for the inhibitory activity of mulberry leaf extract against *S. mutans* is the polyphenol compound and flavonoid constituent of this substance. Kim et al¹³ reported that a mulberry leaf contains a vast quantity of flavonoids and a small quantity of catechin compared with those in green tea. Of these ingredients, flavonoid, as a polyphenol compound in plants, has antioxidant, anti-inflammatory, antimicrobial, and anticarcinogenic properties²⁰. Several researchers reported that flavonoid had antibacterial effect against Gram-positive bacteria^{21,22}.

We suspect that mulberry leaf extract inhibits the growth of *S. mutans* due to its inclusion of a polyphenol compound and flavonoid constituent. However, we used a crude type of mulberry leaf extract in this study. Thus, future studies should purify the extract to isolate the components that are responsible for the observed growth inhibition.

No toxicity should be present in the mulberry leaf extract when the extract is used safely as an antibacterial agent in the oral environment. In this study, we found no statistically significant differences between the treatment with 5 mg/mL of mulberry leaf extract and the control condition with respect to the growth of human gingival fibroblasts. Thus, we can use 5 mg/mL of mulberry leaf extract safely under the oral condition. However, we used only 3 concentrations with wide interval in this study. Therefore, there is a need to find more effective concentration for the inhibition of *S. mutans* using small interval concentrations of mulberry leaf extract.

Lee²³ reported that green tea extract affected normal cell growth at concentrations > 2.5 mg/mL. Compared with

green tea extract, the mulberry leaf extract has lower cytotoxicity at the same concentration, even a side from considerations of effectiveness. However, to compare the effectiveness with other agents, we need to test mulberry leaf extract with other positive controls such as green tea extracts in the future.

Moreover, in future study, the possibility of using mulberry leaf extract for tooth remineralization can be considered, because mulberry leaf contains many minerals including Ca, Fe, Zn, and Mg^{24,25}.

Conclusions

Mulberry leaf extract has been reported to show pharmacological activity. This study examined the inhibition effect of mulberry leaf extract on the growth of *S. mutans*, cariogenic bacteria.

Mulberry leaf extract was obtained with 85% methanol using an ultrasonic method. *S. mutans* KCTC 3065 was used as the experimental bacteria. Agar plate dilution methods were used. The concentrations of mulberry leaf extract used were 5, 20 and 50 mg/mL, and 0 mg/mL was used as the control. The plates were incubated at 37°C for 72 h. The growth inhibition rate and colony-forming units (CFU) were measured. For safety, the cytotoxicity was evaluated by MTT assay on human gingival fibroblasts.

The bacteria treated with mulberry leaf extract decreased over time and treatments with 5, 20 and 50 mg/mL of mulberry leaf extract were associated with higher rates of inhibited growth that was the control condition. Concentrations of mulberry leaf extract of ≥ 20 mg/mL were considered to be cytotoxic.

The results showed that mulberry leaf extract was associated with inhibitory activity with respect to *S. mutans*, suggesting that mulberry leaf extract may be a useful natural agent for the prevention of dental caries.

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