

Growth inhibition of oral disease-causing *Streptococcus* Sp. via methanol extract of lotus (*Nelumbo nucifera*) leaf.

Haeng-Eun Lee¹, Young-Soon Choi^{2*#}, Seoul-Hee Nam^{3*#}

¹Department of Dental Hygiene, Masan University, Changwon-si, 51217, Republic of Korea

²Department of Nursing, College of Health Sciences, Kangwon National University, Samcheok-si, 25949, Republic of Korea

³Department of Dental Hygiene, College of Health Sciences, Kangwon National University, Samcheok-si, 25949, Republic of Korea

#These authors contributed equally to this work

Abstract

We conducted to evaluate the antimicrobial effect of the *Nelumbo nucifera* leaf extract on oral disease-inducing bacteria. The strains used in this experiment were investigated for nine types of *Streptococcus* strains. The antimicrobial activity of *Nelumbo nucifera* leaf extract was measured by the paper disk agar method and minimal inhibitory concentration (MIC). As a result, the methanol extract of *Nelumbo nucifera* leaf showed the strongest antimicrobial activity against *S. anginosus* and *F. nucleatum*, and the weakest activity against *S. oralis* and *S. gordonii*. Methanol extract was absorbed into the paper disk (Φ 8 mm) and the diameter (mm) of the clear zone was measured. The result of the MIC was nearly consistent with the result of the antimicrobial activity that was measured via the paper disk method in that the antimicrobial activity against *S. anginosus* was the strongest, while the antimicrobial activity against *S. gordonii* was the weakest. Therefore, *Nelumbo nucifera* leaf extract can be used effectively in the prevention of oral diseases.

Keywords: Antimicrobial effect, Dental caries, Lotus (*Nelumbo nucifera*) leaf, Oral diseases, *Streptococcus mutans*.

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Introduction

The oral cavity is the first passage to the digestive tract with functions of ingesting, chewing, and sending food down to the pharynx. It also plays an important role of producing sounds and tasting food. Since it is directly exposed to the outside elements, it is invaded by microorganisms all the time. For this reason, the oral cavity creates an environment that is suitable for bacterial growth both nutritionally and physiologically, thereby providing an ideal place for many bacteria to thrive in [1]. Overall, more than 30 species of bacteria are separated in the oral cavity, and the types and ratios of oral bacteria are constantly changing depending on each individual's age, health status, dietary status, and hygiene status [2]. Oral diseases refer to all diseases (e.g., dental caries, periodontal diseases, and stomatitis) that develop inside the mouth. Dental caries and periodontal diseases are major oral diseases that threaten the oral health of Korean people [3,4]. In particular, dental caries is one of the most typical oral diseases that is infectious and accompanied by tooth decay. It is a disease caused by interactions of bacteria in the biofilm on the tooth surface with food and saliva. *Streptococcus mutans* (*S. mutans*) is known as a major causative organism among the bacterial biofilm on the

tooth surface [5]. In addition to *S. mutans*, there are many bacteria that belong to *Streptococcus* sp. that can cause dental caries in the mouth.

In recent years, the development of antimicrobial substances using natural products (e.g., *Nelumbo nucifera*, *Phellodendri cortex*, magnolia bark, and aloe) has been reported [5-8]. Among the natural products used, the antimicrobial effect of the *Nelumbo nucifera* leaf extract has been reportedly limited to food-related microorganisms [8]. However, its effect on bacteria related to dental caries has not been examined at all. Therefore, in this study, the antimicrobial effect of the *Nelumbo nucifera* leaf extract on oral disease-inducing bacteria was investigated for nine types of *Streptococcus* strains, including *Fusobacterium nucleatum*, which is highly related to oral diseases.

Materials and Methods

Sample extraction

For this experiment, 500 g of dried *Nelumbo nucifera* leaf grown in Yeongcheon, Gyeongbuk, South Korea was

purchased from Busan Hyundai Pharm Co., Ltd. After adding 70% methanol 10 times to 100 g of crushed *Nelumbo nucifera* leaf, the extraction was done in a heating mantle at 65°C for 3 h. The extract was filtered 3 times by using filter paper (Advantec No. 2, Toyo, Japan), and the *Nelumbo nucifera* leaf extract was concentrated and lyophilized by using a rotary vacuum evaporator (N-N series, EYELA Co., Japan), an aspirator (A-3S, EYELA Co., Japan), and a freeze dryer (Ilshin Lab. Co., Korea). The lyophilized sample was dissolved in 10% dimethyl sulfoxide (DMSO) and stored at -20°C after dilution.

Experimental strains and culture

The strains used in this experiment were purchased from the Department of Biology at Yonsei University (Table 1) and they were used for the experiment after the subculture in the culture broth, which was anaerobically incubated in the brain-heart infusion (BHI) broth at 37°C for 24 h.

Table 1. List of microbial strains used for antimicrobial activity.

Strains
<i>Streptococcus mutans</i> KCTC 3065
<i>Streptococcus gordonii</i> KCTC 3286
<i>Streptococcus sanguinis</i> ATCC 10556
<i>Streptococcus sobrinus</i> KCTC 3288
<i>Streptococcus rattii</i> KCTC 3294
<i>Streptococcus anginosus</i> KCTC 3327
<i>Streptococcus criceti</i> KCTC 3292
<i>Streptococcus oralis</i> ATCC 55229
<i>Fusobacterium nucleatum</i> KCTC 2640

Paper disk method

The antimicrobial activity of *Nelumbo nucifera* leaf extract was measured by using the paper disk agar method [9]. One loop of each of the nine experimental strains was inoculated into 10 ml BHI broth and anaerobically incubated at 37°C for 24 h. First, 15 ml of the sterilized BHI medium was dispensed into a petri dish and solidified, and then the culture broth of each strain was uniformly smeared with a sterilized cotton swab. Meanwhile, the *Nelumbo nucifera* leaf extract was completely dissolved in 10% DMSO and diluted to make a maximum concentration of 10 mg/ml. The 50 µl diluted extract at each concentration was absorbed in a paper disk (Φ 8 mm), and then the disk was dried. The dried disk was placed on the surface of the test plate media, adhered, and anaerobically incubated at 37°C for 24 h. The clear zone (mm) around the paper disk was measured in order to examine the antimicrobial activity of the extract.

Measurement of minimal inhibitory concentration of the *Nelumbo nucifera* leaf extract

In order to measure the minimal inhibitory concentration (MIC), the broth dilution method [10] was used. Each sample strain was inoculated into a BHI broth (10 ml) and anaerobically incubated at 37°C for 24 h. Then, 50 µl of each cultured strain (1×10^7 CFU/ml) was inoculated into a 96-microwell plate containing the *Nelumbo nucifera* leaf extract at each concentration (0 mg/ml, 2.125 mg/ml, 4.25 mg/ml, 8.5 mg/ml, 17 mg/ml, and 34 mg/ml). This microwell plate was anaerobically incubated at 37°C for 24 h, and then the absorbance (OD) was measured at an absorption wavelength of 490 nm with an ELISA reader in order to evaluate the antimicrobial effect of the *Nelumbo nucifera* leaf extract. Herein, the minimum concentration of the sample, in which the growth of each strain has not been observed, was defined as the MIC.

Results

Measurement of antimicrobial activity via paper disk method

The antimicrobial effect of *Nelumbo nucifera* leaf extract on each oral bacteria that was examined by measuring the clear zone via the paper disk method is presented in Table 2. The clear zone of *S. mutans* was found to be 1.25 mm and 2 mm at 5 mg/ml and at 10 mg/ml, respectively. The clear zone of *S. gordonii* was 2 mm at 10 mg/ml, while the clear zone of *S. sanguinis* was 1.5 mm, 2 mm, and 4.5 mm at 2.5 mg/ml, 5 mg/ml, and 10 mg/ml, respectively. The clear zone of *S. sobrinus* was found to be 1.5 mm and 5.5 mm at 5 mg/ml and 10 mg/ml, respectively, while the clear zone of *S. rattii* was 1.5 mm and 2 mm at 5 mg/ml and 10 mg/ml, respectively. The clear zone of *S. anginosus* was 7 mm, 9 mm, and 11 mm at 2.5 mg/ml, 5 mg/ml, and 10 mg/ml, respectively, thereby showing the highest antimicrobial effect. The clear zones of *S. criceti* and *S. oralis* were 2.5 mm and 1.5 mm at 10 mg/ml, respectively, while the clear zone of *Fusobacterium nucleatum* was 6 mm, 7.5 mm, and 10.5 mm at 2.5 mg/ml, 5 mg/ml, and 10 mg/ml, respectively.

Table 2. Antibacterial activity of 70% methanol extract of *Nelumbo nucifera* via paper disk method.

Strains	Clear Zone (mm)				
	0	1.25	2.5	5	10
<i>Streptococcus mutans</i>	-	-	-	1.25	2
<i>Streptococcus gordonii</i>	-	-	-	-	2
<i>Streptococcus sanguinis</i>	-	-	1.5	2	4.5
<i>Streptococcus sobrinus</i>	-	-	-	1.5	5.5
<i>Streptococcus rattii</i>	-	-	-	1.5	2
<i>Streptococcus anginosus</i>	-	-	7	9	11
<i>Streptococcus criceti</i>	-	-	-	-	2.5

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<i>Streptococcus oralis</i>	-	-	-	-	1.5
<i>Fusobacterium nucleatum</i>	-	-	6	7.5	10.5

Therefore, the methanol extract of *Nelumbo nucifera* leaf showed the strongest antimicrobial activity against *S. anginosus* and *F. nucleatum*, and the weakest activity against *S. oralis* and *S. gordonii* (Figures 1 and 2). Methanol extract was absorbed into the paper disk (Φ 8 mm) and the diameter (mm) of the clear zone was measured.

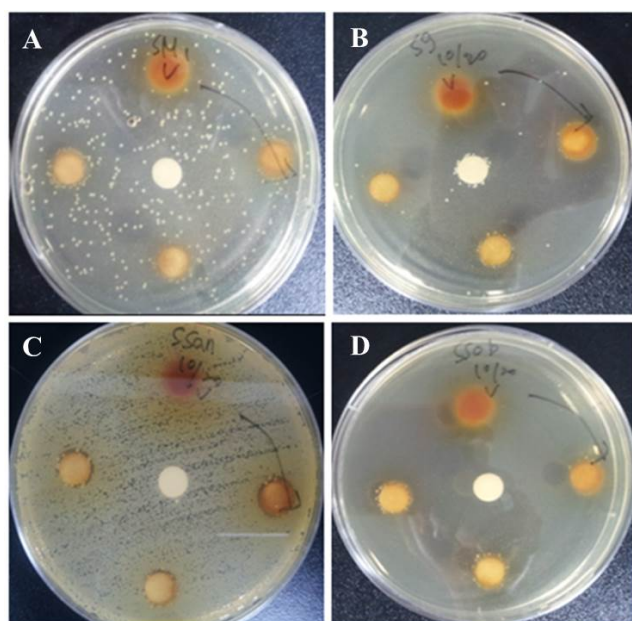


Figure 1. Antibacterial activity of the methanol extract of *Nelumbo nucifera* on the growth of (A) *Streptococcus mutans*, (B) *Streptococcus gordonii*, (C) *Streptococcus sanguinis*, and (D) *Streptococcus sobrinus* via paper disk method; A) 10 mg/ml; B) 5 mg/ml; C) 2.5 mg/ml; D) 1.25 mg/ml.

Measurement of the minimal inhibitory concentration of *Nelumbo nucifera* leaf extract

The antimicrobial effect of *Nelumbo nucifera* leaf extract on each strain that was measured with the MIC is presented in Table 3. The MIC of the extract against *S. anginosus* was 2.13 mg/ml, which was the strongest antimicrobial activity, while the MIC against *S. sanguinis*, *S. sobrinus*, and *F. nucleatum* was 4.25 mg/ml, respectively, and the MIC against *S. mutans*, *S. gordonii*, *S. criceti*, *S. ratti*, and *S. oralis* was 8.5 mg/ml, respectively. The result of the antimicrobial activity that was measured with the MIC was nearly consistent with the result of the antimicrobial activity that was measured via the paper disk method in that the antimicrobial activity against *S. anginosus* was the strongest, while the antimicrobial activity against *S. gordonii* was the weakest.

Table 3. Minimum inhibitory concentrations of 70% methanol extract *Nelumbo nucifera*.

Strains	Concentration (mg/ml)						MIC (mg/ml)
	0	2.13	4.25	8.5	17	34	
<i>Streptococcus mutans</i>	+	+	+	-	-	-	8.50
<i>Streptococcus gordonii</i>	+	+	+	-	-	-	8.50
<i>Streptococcus sanguinis</i>	+	+	-	-	-	-	4.25
<i>Streptococcus sobrinus</i>	+	+	-	-	-	-	4.25
<i>Streptococcus ratti</i>	+	+	+	-	-	-	8.50
<i>Streptococcus anginosus</i>	+	-	-	-	-	-	2.13
<i>Streptococcus criceti</i>	+	+	+	-	-	-	8.50
<i>Streptococcus oralis</i>	+	+	+	-	-	-	8.50
<i>Fusobacterium nucleatum</i>	+	+	-	-	-	-	4.25

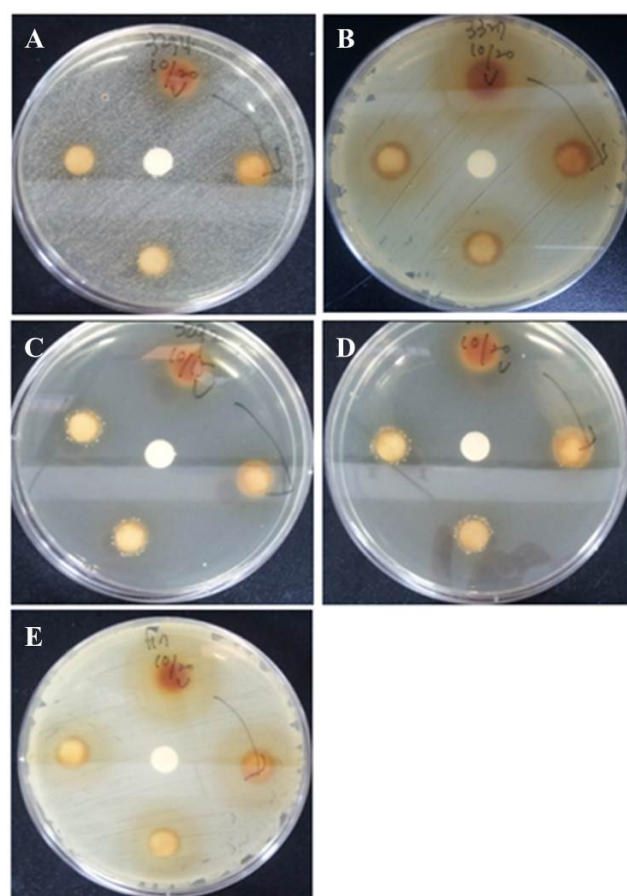


Figure 2. Antibacterial activity of the methanol extract of *Nelumbo nucifera* on the growth of (A) *Streptococcus ratti*, (B) *Streptococcus anginosus*, (C) *Streptococcus criceti*, (D) *Streptococcus oralis*, and (E) *Fusobacterium nucleatum* via paper disk method; A) 10 mg/ml; B) 5 mg/ml; C) 2.5 mg/ml; D) 1.25 mg/ml; E) 0 mg/ml.

Discussion

The oral cavity is one of the parts of the body where oral diseases and systemic diseases are frequently caused by pathogenic infectious strains and resident flora by providing a remarkably easy environment for various microorganisms to thrive in [11]. Methods for oral health improvement by controlling the growth conditions of the oral microorganisms

have been diversely studied and developed. The misuse and abuse of drugs and the drug resistance to nosocomial pathogens have recently become serious problems; therefore, the need for new alternative drugs is becoming apparent [12]. As a result, there is a recent growing interest in studies on the inhibition of oral diseases using natural products without side effects [11]. Natural products have been used in folk remedies for thousands of years, and there is a growing interest in the development of natural antimicrobials and physical health as of late [13,14]. The purpose of this study was to investigate the effect of the methanol extract of *Nelumbo nucifera* leaf, which is a natural product, on oral bacteria. The antimicrobial activity against oral bacteria was confirmed by treating nine types of strains with the methanol extract of *Nelumbo nucifera* leaf.

In terms of the MIC against *S. mutans* using a natural product, the MIC of gold was 125 mg/ml in the study of Paek et al. [15], the MIC of Schisandra chinensis was 50.0 mg/ml in the study of Chung et al. [16], and the MIC of cassia seed was 5.0 mg/ml in the study of Na et al. [17]. Since the MIC of *Nelumbo nucifera* leaf extract is 8.5 mg/ml against *S. mutans*, its antimicrobial effect is considered to be relatively strong, as compared to those of the other natural products. This result was similar to that of the study showing that the MIC of *Nelumbo nucifera* leaf extract against *S. mutans*, *F. nucleatum*, and *A. actinomycetemcomitans* was as low as 625 µg/mL, thereby demonstrating the superiority of *Nelumbo nucifera* leaf extract with regard to its antimicrobial effect [18]. Based on these results, it has been confirmed that the methanol extract of *Nelumbo nucifera* leaf has an excellent antimicrobial effect against many dental caries-causing bacteria, and it can be developed as a highly stable and natural agent for dental caries prevention.

Conclusion

Nelumbo nucifera leaf extract showed an excellent antimicrobial activity against dental caries-causing bacteria. Therefore, it is considered as an effective agent for the prevention of dental caries and it can be used as an alternative for the existing chemical agents. It is believed that *Nelumbo nucifera* leaf extract can be used effectively in the prevention of oral diseases (e.g., dental caries), and ultimately contribute to the improvement of oral health promotion in the future.

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***Correspondence to**

Young-Soon Choi

Department of Nursing

College of Health Sciences

Kangwon National University

Republic of Korea

Seoul-Hee Nam

Department of Dental Hygiene

College of Health Sciences

Kangwon National University

Republic of Korea