ANTIMICROBIAL ACTIVITY OF ARTEMISIA SPECIES AGAINST CLINICALLY ISOLATED *STREPTOCOCCUS MUTANS*

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Abstract

Streptococcus mutans plays a major role in the formation of dental plaque and it is considered one of the important pathogens in the development of dental caries. Established dental plaque can be more resistant to antimicrobial agents and offers nutrient rich and relatively stable cariogenic conditions for bacteria. Thus bacteria growing in dental plaque have strong resistance to antimicrobial agents and they are not removed easily by the flow of saliva. Many researchers have been performed using natural materials, especially herbal extracts to prevent dental plaque. However, the strains of mutans streptococci used in the researches were not from Koreans. Therefore, it would be necessary to evaluate the antimicrobial activity of herbal extracts against clinical isolates of *Streptococcus mutans* isolated from saliva of Koreans living in Jeollabuk-do.

For this study four clinical isolates were isolated from saliva samples of seventeen Korean people to investigate whether essential oils of *Artemisia spp.* have antimicrobial activity against them including four reference strains of *Streptococcus mutans*. Minimum inhibitory concentrations, Minimum bactericidal concentrations and time kill studies were performed and the results showed that *Artemisia lavandulaefolia DC*, *Artemisia scoparia*, and *Artemisia capillaries* have antimicrobial activity against the *Streptococcus mutans* clinical isolates and reference strains

Key words : Artemisia, Antimicrobial agent, *Streptococcus mutans*

I. Introduction

Streptococcus mutans (*S. mutans*) is a Gram-positive, facultatively anaerobic bacteria which plays a major role in the formation of dental plaque and it is considered one of the important pathogens in the development of dental caries[1-3]. Initial adherence of *S. mutans* to the tooth surface occurs in a sucrose-independent way by Antigen I/II (Ag I/II)[4] and glucans synthesized by glucosyltransferases of *S. mutans* in a sucrose dependent way, which mediate attachment of bacteria to the tooth surface and form dental plaque[5-7], a complex microbial biofilm which is a well organized, cooperating community of microorganisms[8,9]. Once *S. mutans* attaches to the surface of the tooth, it produces acid, lactic acid and it demineralizes tooth enamel inducing dental decay[10]. Glucan-dependent adherence and accumulation by mutans streptococci is critical for the plaque formation[11,12] and a well established dental plaque, biofilm, can be more resistant to antimicrobial agents and offers nutrient rich and relatively stable cariogenic conditions for bacteria. Thus bacteria growing in dental plaque have

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strong resistance to antimicrobial agents and they are not removed easily by the flow of saliva so tooth-brushing, flossing and using a mouthwash containing antimicrobial agents would be good methods to prevent dental plaque.

The effects of antimicrobial agents such as Chlorhexidine[13], Alkylamine salts and amides[14], quaternary ammonium salts[15], phosphorylated polyethylene glycol[16-18] and herbal extracts have long been studied to reduce dental plaque. However, the strains of mutans streptococci used in the researches were not from Koreans. Therefore, it would be necessary to evaluate the antimicrobial activity of herbal extracts against clinical isolates of mutans streptococci, especially *S. mutans*, isolated from saliva of Koreans.

Artemisia is used for antioxidant, therapeutic applications and also have been suggested that it is an effective antimicrobial agent[19,20], but there are only few studies on its antimicrobial activity against *S. mutans* and the strains used in the researches were not clinical isolates isolated from Koreans but reference strains.[21]

Three species of the genus *Artemisia* were used in this study; *Artemisia lavandulaefolia DC* (*A. lavandulaefolia*), *Artemisia scoparia* (*A. scoparia*) and *Artemisia capillaries* (*A. capillaries*). *A. scoparia* is used for mosquito repellent and has hepatoprotective effects against carbon tetrachloride[22,23]. *A. capillaries* is used for herbal medicine and inhibits the activity of herpes simplex virus[24,25].

The purpose of the present study was to isolate *S. mutans* clinical isolates (Ch, P, L1 and L2) from Korean patients with more than one dental caries and investigate antimicrobial activity of *A. lavandulaefolia DC*, *A. scoparia* and *A. capillaries* against the *S. mutans* clinical isolates including reference strains, KCTC 3298, KCTC 3605 (ATCC 25175), KCTC 3306 and GS5 with Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and time-kill studies.

Ⅱ. Material and Methods

1. Bacterial strains

1-1. *Streptococcus* strains

Streptococcus mutans GS5 was kindly provided by F. Macrina, Virginia Common Wealth University, Richmond, VA, USA. The other reference strains were KCTC 3298, KCTC 3605 (ATCC 25175), KCTC 3306. The four clinically isolated strains were Ch, P, L1 and L2.

1-2. Isolation of *S. mutans*

Saliva samples were collected from 17 patients (aged 6-7 years) who had more than 1 dental caries in 50 ml conical tubes and stored at 4℃ before analysis. The samples were serially diluted 10-fold twice and 100 ul of each dilution was transferred onto a selective medium of *S. mutans*, medium plates (90 g Mitis salivarius agar and 100 g sucrose in 1L distilled water, autoclave and cooling to 50℃, then 0.2 g sulfisoxazole, 300 units bacitracin and 10 mg valinomycin) and grown at 37℃ in an anaerobic chamber for 16 hrs. *S. mutans* exhibit a distinct colonial morphology on mitis salivarius agar[26]. Mitis salivarius agar was originally devised to isolate fecal streptococci but it has mostly used for the isolation of oral streptococci, including *S. mutans*, because of its selective and differential properties.

The samples were cultured selectively on MS-MUTV medium and brain heart infusion (BHI) medium plates. After several rounds of selection a colony from each plate was inoculated into BHI broth and grown at 37℃ in an anaerobic chamber for 16 hrs and qualified at PCR screening using PCR primers for S. mutans AgI/II gene (also known as protein B, P1, Pac, and SpaP).

1-3. PCR screening

The PCR reaction mixture (50 ㎕) contained Taq polymerase, dNTPs mixture (dATP, dCTP, dGTP and dTTP), primers (Ag I/II F; 5′-ACATGCATGCGGACAAAAGGTTTTGCCGATG-3′ and Ag I/II MR; 5′-GTATACCAGAGCTAGCGAACCGGGATC-3′) and distilled water. The amplification reaction was 5 minute denaturation at 95℃, followed by 30 cycles of denaturation at 95℃ for 1 minute, annealing at 55℃ for 1 minute and extension at 72℃ for 1 minute with a final extension at 72℃ for 5 minutes.

2. Extraction of *Artemisia spp.*

Essential oils of *Artemisia spp.* (*A. lavandulaefolia DC*, *A. scoparia*, and *A. capillaries*) were supplied by Cha et al[27] and Cha et al[28]. In brief, essential oils were prepared by distillation of air-dried *Aretemisia* with a modified Clevenger-type apparatus.

3. Antibiotic

Ampicillin was used to compare the sensitivity with the essential oils.

4. MICs and MBCs

MICs and MBCs are the tests to monitor the activity of antimicrobial agents[29]. MIC is defined as the lowest concentration of antibiotic sufficient to inhibit bacterial growth when tested *in vitro* or as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. MBC is defined as the lowest concentration of antibiotic required to kill an organism.

MICs of the essential oils of *A. lavandulaefolia DC, A. scoparia* and *A. capillaries* were defined by broth dilution method. Two fold dilutions were made for the test. The initial concentration of *A. lavandulaefolia DC* and *A. scoparia* was 25.6 ug/mL and 102.4 ug/mL for *A. capillaries*, respectively. About 1×10^8 CFU/ml of each strain was inoculated into 96 well containing BHI broth and the essential oils and grown at 37℃ in an anaerobic chamber. After 16 hrs, the presence or absence of turbidity was evaluated. Optical Density (OD) of each well was measured at 550 nm using spectrophotometer (Optizen 2120uv, Mecasys, Korea). The lowest concentration that did not show growth was determined as the minimum inhibitory concentration. MICs were tested in quadruplicate and results are presented as the means of them.

MBCs were the lowest concentration of the essential oils that prevented the growth of the *S. mutans* strains following broth dilution procedures. The cells in the wells of 0.5 × MIC, 1 × MIC, 2 × MIC, 4 × MIC, and 8 × MIC were transferred onto the BHI plates. The dishes were incubated for 16 hrs at 37℃ in the anaerobe chamber. The minimum concentration showing no growth on the plates was determined as the MBC of the essential oil. MBCs were tested in quadruplicate and results are the means of them.

5. Time-kill studies

Time-kill studies are generally used in investigations of antimicrobial activity of antimicrobial agents[30,31]. *A. lavandulaefolia DC* and ampicillin were chosen for time-kill studies. Viability counts of *A. lavandulaefolia DC* and ampicillin-containing *S. mutans* strains in BHI broth were performed at 0.5 hr, 1 hr, 2.5 hrs and 4 hrs by plating onto BHI plate. The plates were incubated at 37℃ in the anaerobic chamber for 16 hrs. Time-kill activities were measured in duplicate and A. lavandulaefolia DC and ampicillin concentration chosen was 1 × MIC for each strain. Results are the means of two studies.

Ⅲ. Results

1. PCR screening to select clinical isolates

Clinical isolates were from saliva samples collected from Korean patients who had more than one dental caries. The isolates were selected based on the colony morphology on MS-MUTV agar plate and screened by PCR (Fig. 1) using PCR primers of *S. mutans* AgI/II gene. Four clinical isolates (Ch, L1, L2, and P) were selected from seventeen samples.

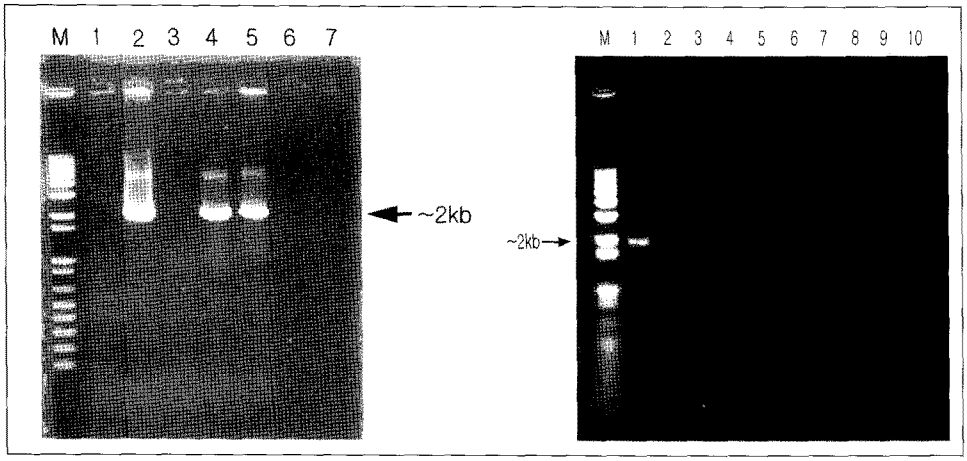

Fig. 1. PCR screening to select clinical isolates of *S. mutans*. The colonies from 17 patients were selected and qualified at PCR screening using primers of AgI/II gene, Ag I/II F and Ag I/II MR, and 4 of them were identified as *S. mutans* (M: marker).

2. Susceptibility

The selected clinical isolates were tested for antimicrobial activity of *A. lavandulaefolia DC*, *A. scoparia* and *A. capillaris* with four reference strains. MICs and MBCs of the essential oils of the *Artemisia spp.* are shown in Table 1. According to Table 1, *A. lavandulaefolia DC*, *A. scoparia* and *A. capillaris* have antimicrobial activity against all *S. mutans* strains.

A. lavandulaefolia DC MICs were the same as 0.8 ug/mL for reference strains (Fig. 2) and ranging between 0.4 and 0.8 ug/mL for clinical isolates (Fig. 3). *A. scoparia* MICs were 0.8 to 1.6 ug/mL for reference strains (Fig. 4) and 0.4 to 0.8 ug/mL for clinical isolates (Fig. 5). *A. capillaries* MICs were 0.2 to 25.6 ug/mL for reference strains (Fig. 6) and 1.6 to 3.2 ug/mL for clinical isolates (Fig. 7).

A. lavandulaefolia DC MBCs were 1.6 to 6.4 ug/mL for reference strains and 0.8 to 3.2 ug/mL for clinical isolates. *A. scoparia* MBCs were 1.6 to 12.8 ug/mL for reference strains and 0.8 to 3.2 ug/mL for clinical isolates. A. capillaries MBCs for KCTC 3306, KCTC 3605, KCTC 3298, and GS5 were not possible to read and were 6.4 to 12.8 ug/mL for clinical isolates.

Table 1. MICs and MBCs of *A. lavandulaefolia DC*, *A. scoparia*, *A. capillaries*, and ampicillin for *S. mutans* four reference strains (KTCC 3306, KTCC 3605, KTCC 3298 and GS5) and four clinical isolates (P, L1, L2, and Ch) (ug/mL)

Strains/agents	*Artemisia lavandulaefolia DC*		*Artemisia scoparia*		*Artemisia capillaries*		*Ampicillin*	
	MICs	MBCs	MICs	MBCs	MICs	MBCs	MICs	MBCs
3306	0.8	1.6	0.8	6.4	12.8	↑	1	1
3605	0.8	6.4	1.6	1.6	12.8	↑	1	1
3298	0.8	6.4	1.6	6.4	0.2	↑	1	1
GS5	0.8	6.4	0.8	12.8	25.6	↑	1	1
P	0.8	3.2	0.4	0.8	3.2	12.8	1	1
L1	0.4	3.2	0.8	3.2	3.2	6.4	1	1
L2	0.4	3.2	0.8	3.2	3.2	6.4	1	1
Ch	0.4	0.8	0.4	0.8	1.6	6.4	1	1

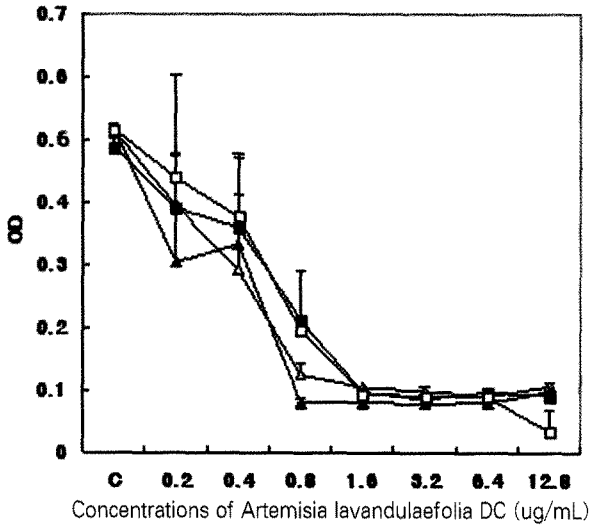

Fig. 2. *A. lavandulaefolia DC* MICs against *S. mutans* reference strains. KTCC 3605 (△), KTCC 3298 (■), KTCC 3306 (▲) and GS 5 (□); Optical Density was measured at 550 nm. The results are mean values SD from four tests. C: control

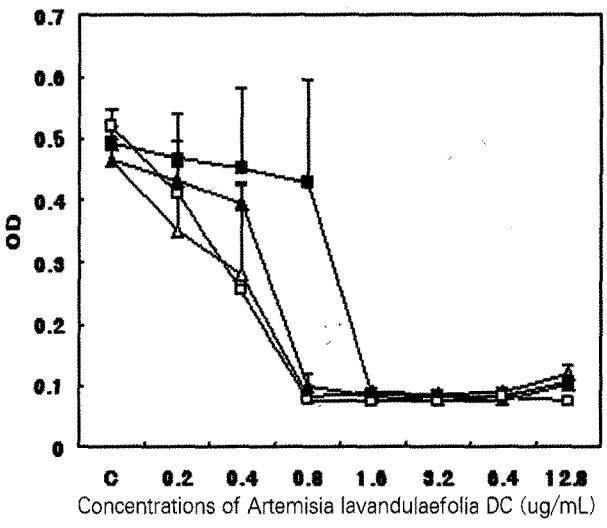

Fig 3. *A. lavandulaefolia DC* MICs against *S. mutans* clinical isolates; Ch (△), P (■), L1 (▲) and L2 (□). Optical Density was measured at 550nm. The results are mean values SD from four tests. C: control.

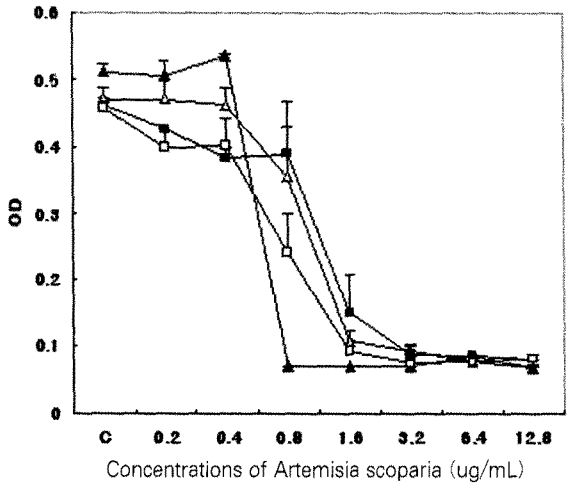

Fig. 4. *A. scoparia* MICs against *S. mutans* reference strains; KTCC 3605 (△), KTCC 3298 (■), KTCC 3306 (▲) and GS 5 (□). Optical Density was measured at 550 nm. The results are mean values SD from four tests. C: control.

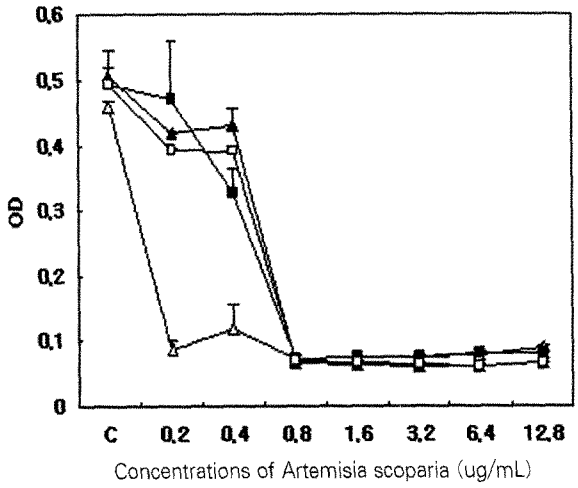

Fig. 5. *A. scoparia* MICs against *S. mutans* clinical isolates; Ch (△), P (■), L1 (▲) and L2 (□). Optical Density was measured at 550 nm. The results are mean values SD from four tests. C: control.

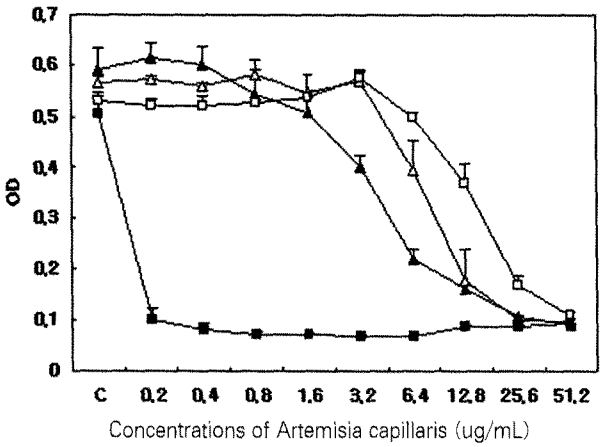

Fig. 6. *A. capillaries* MICs against *S. mutans* reference strains; KTCC 3605 (△), KTCC 3298 (■), KTCC 3306 (▲) and GS 5 (□). Optical Density was measured at 550 nm. The results are mean values SD from four tests. C: control.

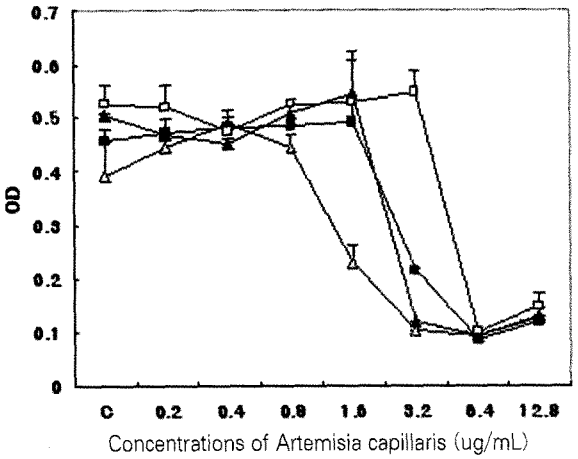

Fig. 7. *A. capillaries* MICs against *S. mutans* clinical isolates; Ch (△), P (■), L1 (▲) and L2 (□). Optical Density was measured at 550 nm. The results are mean values SD from four tests. C: control.

3. Time-kill studies

According to the Time-kill studies, *A. lavandulaefolia* DC killed 100% of KCTC 3298 and almost 100% of GS5 at 4 hrs (data not shown) and between 99% and 99.90% of other strains at 0.5 hr and killed > 99.99% of KCTC 3298 and GS5 at 0.5 hr (data not shown). All the strains were killed between 99.90% and 99.99% at 2.5 hrs (Fig. 8). Thus *A. lavandulaefolia DC* was bactericidal for all the *S. mutans* strains at 1 × MIC after 0.5 hr. Ampicillin was used as a standard antibiotic to compare the sensitivity of the essential oil of *A. lavandulaefolia DC* (Fig. 9).

The results of MICs, MBCs of the essential oils of *A. lavandulaefolia DC, A. scoparia* and *A. capillaries* and the time-kill studies of *A. lavandulaefolia DC* showed that they had antimicrobial activity against all the *S. mutans* strains tested in this study.

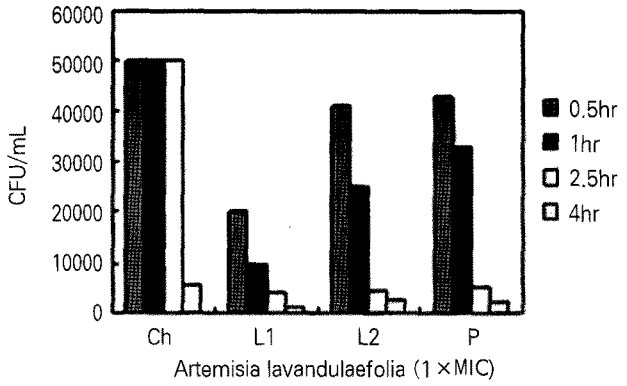

Fig. 8. The results of time-kill studies of *A. lavandulaefolia DC* against clinical isolates. The No. is CFU/mL and the concentration of *A. lavandulaefolia DC* was 1 × MIC. Viability counts of *A. lavandulaefolia DC*-containing *S. mutans* suspensions were performed at 0.5 hr, 1 hr, 2.5 hrs and 4 hrs by plating 100 ul from each suspension. The initial concentration of *S. mutans* was 1 x 10^8 CFU/mL.

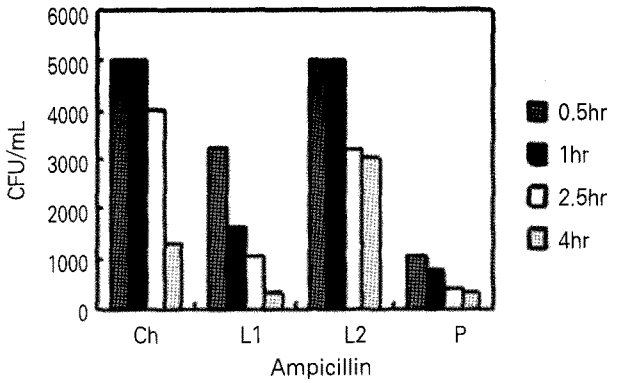

Fig. 9. The results of time-kill studies of ampicillin against clinical isolates. The No. is CFU/mL and the concentration of ampicillin was 1 × MIC (1 ug/mL). Viability counts of ampicillin-containing *S. mutans* suspensions were performed at 0.5 hr, 1 hr, 2.5 hrs and 4 hrs by plating 100 ul from each suspension. The initial concentration of *S. mutans* was 1 × 10^8 CFU/mL.

Ⅳ. Discussion

S. mutans was isolated from human carious lesions in 1924, confirmed by a number of investigators and also found that S. mutans is related with the development of dental caries[32,33]. Clinical observations in humans and animals indicate that plaque formation is an essential requirement for both dental caries and periodontal disease. *S. mutans* comprises the primary component of dental plaque, metabolizes exogenous dietary carbohydrates including sucrose and makes lactic acid[9]. The accumulation of lactic acid in the plaque biofilm results in a localized drop in pH and subsequent demineralization of tooth enamel. Therefore, there have been researches on elimination of dental plaque with antimicrobial agents[34].

There are a number of studies to evaluate the antimicrobial activity of herbal extracts such as Eucalyptol and Thymol[35], Streblus asper[36], garlic and tea tree oil[37], green tea[38] and Uncaria tomentosa against *S. mutans*[39]. The purpose of this study was to isolate *S. mutans* from saliva samples of Koreans and investigate whether the essential oils of *A. lavandulaefolia DC*, *A. scoparia*, and *A. capillaries* have antimicrobial activity against them including reference. According to a study used the extract of *Artemisia spp.*, *Mugwort*, on *S. mutans*[21], the MIC was 0.5 mg/mL against a reference strain, KCTC 5316 and this is much higher than the MICs of the essential oils of *A. lavandulaefolia DC*, *A. scoparia*, and *A. capillaries* used in this study against reference strains; 0.8 ug/mL, 0.8 to 1.6 ug/mL and 0.2 to 25.6 ug/mL, respectively and also higher than the MICs against clinical isolates; 0.4 to 0.8 ug/mL, 0.4 to 0.8 ug/mL and 1.6 to 3.2 ug/mL, respectively (Table 1). It may suggest that *A. lavandulaefolia DC*, *A. scoparia*, and *A. capillaries* have more antimicrobial activity than Mugwort against *S. mutans*. The MICs of A. capillaries were higher (1.6 to 3.2 ug/mL against clinical isolates and 0.2 to 25.6 ug/mL against reference strains) than the MICs of *A. lavandulaefolia DC* and *A. scoparia* (0.4 to 0.8 ug/mL and 0.4 to 0.8 ug/mL against clinical isolates and 0.8 ug/mL and 0.8 to 1.6 ug/mL against reference strains, respectively). The MBCs of the essential oils of *A. capillaries* (6.4 to 12.8 ug/mL) were slightly higher than the MBCs of *A. lavandulaefolia DC* and *A. scoparia* (0.8 to 3.2 ug/mL) for clinical isolates. These results suggest that *A. lavandulaefolia DC* and *A. scoparia* have more antimicrobial activity than *A. capillaries* against *S. mutans*.

The essential oil of *A. lavandulaefolia DC* could kill *S. mutans* clinical isolates within 0.5 hr (90% - 99%) at 1 × MICs (0.8 ug/mL for P and 0.4 ug/mL for Ch, L1 and L2, respectively). The time-kill studies also indicate that the essential oil of *A. lavandulaefolia DC* is bactericidal against the *S. mutans* clinical isolates.

In conclusion, according to the results of MICs, MBCs and time-kill studies, the essential oils of *A. lavandulaefolia DC*, *A. scoparia*, and *A. capillaries* have antimicro-

bial activity against the *S. mutans* clinical isolates including reference strains and this suggests that they could be used to control dental plaque formation and subsequent dental caries formation.

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국문초록

임상 분리된 *Streptococcus mutans*에 대한 Artemisia species의 항균 활성도

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*Streptococcus mutans*는 Mutans streptococci 그룹에 속하는 치아우식 유발균으로 알려져 있다. 국내 자생쑥 종의 추출물이 이러한 치아우식을 유발하는 *Streptococcus mutans*균에 대해 활성 억제 효과가 있다는 것이 표준균주를 이용한 실험에서 밝혀져 있으며, 본 연구에서는 환자에서 분리된 S. mutans를 중심으로 쑥의 항균 효과를 알아보았다.

네 가지 임상 분리균주; Ch, L1, L2, P와 네 가지 표준균주; KCTC (Korean Culture for Type Collection) 3298, KCTC 3605 (ATCC 25175), KCTC 3306, *Streptococcus mutans* GS5를 이용하여 세 가지 종류의 쑥; 참쑥(*Artemisia lavandulaefolia DC*), 비쑥(*Artemisia scoparia*), 사철쑥(*Artemisia capillaries*)의 효능을 검증하였다. 항균활성은 세 가지 종류의 쑥 추출물의 *Streptococcus mutans*에 대한 최소 억제농도(MIC)와 최소살균농도(MBC), time-kill studies를 통해 알아보았다.

최소억제농도는 표준균주에 대해 각각 참쑥이 0.8 ug/mL, 비쑥이 0.8-1.6 ug/mL, 사철쑥이 0.2-25.6 ug/mL이었으며, 분리된 균주에 대해서는 참쑥이 0.4-0.8 ug/mL, 비쑥이 0.4-0.8 ug/mL, 사철쑥 1.6-6.4 ug/mL이었다.

최소살균농도는 표준균주에 대해서 참쑥이 1.6-6.4 ug/mL, 비쑥이 6.4-12.8 ug/m 이였다. 분리균주에 대해서는 참쑥이 0.8-3.2 ug/mL, 비쑥이 0.8-3.2 ug/mL, 사철쑥은 6.4-12.8 ug/mL이었다.

Time-kill assay에서 KCTC 3298이 4 hrs에 100% 살균되었으며, GS5도 거의 100% 살균되었고, L1과 L2, P는 0.5 hrs에 99.90% 살균되었다. 0.5 hrs에 KCTC 3298와 GS5는 99.99% 이상 살균되었고, 모든 균주들이 2.5 hrs에 99.99% 살균되었다. 전체적인 실험 결과 세 가지 종류의 쑥은 표준 균주 및 네 가지 분리 균주 모두에서 항균 효과가 있음을 확인하였다.

주요어 : 쑥, 항균효과, *Streptococcus mutans*